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THE FRACTIONATION OF AUSTRALIAN SNAKE VENOMS

2. THE VENOMS OF THE TIGER SNAKE (*NOTECHIS SCUTATIS*), BLACK TIGER SNAKE (*NOTECHIS SCUTATIS* VAR. *NIGER*), AND BLACK SNAKE (*PSEUDECHIS PORPHYRIACUS*).

by

HENRY FRANCIS HOLDEN

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 23rd January, 1933.)

In an earlier paper (2) were presented the results of the fractionation of death adder venom by extraction with 45% ethyl alcohol. This method was, however, found to effect only incomplete fractionation of venoms containing thrombin. The only instance previously recorded of the separation of the thrombin from the "neurotoxin" of the venom of an Australian snake was due to Martin (3), who, by means of differential ultra-filtration under high pressure, through gelatine membranes supported on filter candles, obtained a separation of these constituents in the venom of the black snake.

Although preferential thermal inactivation of venom thrombins has been observed, it seemed inadvisable to resort to heat for their removal, since heat, sufficiently intense and prolonged to inactivate thrombin, has a deleterious effect on other constituents of venom. In the present work, therefore, filtration and adsorption have been employed; the latter has been found most useful for the preparation of thrombin-free fractions of these venoms.

1. FILTRATION THROUGH PYROXYLIN MEMBRANES.

These experiments were carried out by Miss Mavis Freeman. Death adder venom was used, and the presence in the filtrates of proteose and of coagulable protein determined mainly by chemical tests.

The membranes were made by Adair's (1) method, giving six coats of 3·5% pyroxylin solution; variation of permeability was obtained by altering the times between the application of successive coats, the period of final drying and the percentage of alcohol in which the membranes were then immersed. Five grades of membranes were prepared, as set out in Table 1.

TABLE 1.

Grades of Pyroxylin Membranes used for Filtration at Low Pressures.

Grade of membrane.	Time between the coats.	Period of final drying.	Percentage of alcohol used for immersion.
1	4 minutes	15 minutes	50%
2	3 minutes	10 minutes	50%
3	3 minutes	8 minutes	50%
4	3 minutes	7 minutes	50%
5	2 minutes	5 minutes	80%

At a pressure of 30 cm. of mercury membrane 1 was impermeable to proteins and dextrins, membranes 2, 3, and 4 to proteins but not to dextrins, and membrane 5 was permeable to starch as well as dextrins. At this pressure none of these membranes allowed the passage of any appreciable quantity even of the proteose fraction of death adder venom.

Further experiments were made with membrane 5, using venom dissolved in distilled water, in 0·9% sodium chloride, and in 3·4% sodium chloride. When the pressure was raised to 35 cm. of mercury some venom passed through the filter, but there was no useful separation of the proteose from the coagulable fraction. At 40cm. and 45 cm. pressure the result was the same, and increased amounts of both fractions appeared in the ultra filtrate.

It was clear that ultra-filtration would be of little value for the preparation of large amounts of the fractions of venoms unless more impermeable membranes and higher pressures were used.

Mixtures of proteins are not readily separated by ultra-filtration under moderate pressures, the filtrable constituent tending to drag the normally unfiltrable protein through the filter. It was hoped that this difficulty might be avoided by the use of high pressures, and a number of experiments were performed using the apparatus originally designed by Martin (3).

2. MARTIN'S METHOD.

Martin's original apparatus was in the Department of Physiology in the University of Melbourne, and, through the courtesy of Professor W. A. Osborne, it was made available for this work.

The membranes were made of Coignet's gold label gelatine, containing 15·5% water and 2·06% ash. Fifty c.c. of solution was made by dissolving the necessary weighed quantity in distilled water at 60°C. The chamber containing the filter candle was warmed to 60°C., filled with the solution of gelatine, and a pressure of 300 to 350 lb. per square inch at once applied. After about 5 c.c. of gelatine solution had passed through the filter the pressure was released, and the chamber and filter placed in a refrigerator at 0°C. for a period of seasoning. The cylinder was then opened and the residual gelatine removed immediately before the introduction of the solution of venom.

0·2 grm. black snake venom, dissolved in 20 c.c. of 0·9% sodium chloride solution, was placed in the chamber, and a pressure of 350 to 400 lb. per square inch applied for 24 hours. The chamber was then filled up with 0·9% sodium chloride, and pressure applied for a further period of 24 hours. The pressure was then released, and the filtrates united and made up to 40 c.c. The chamber was emptied and washed out with 0·9% sodium chloride,

and the residual fluid and washings made up to 20 c.c. By variation of the concentration of gelatine and the time of seasoning much variation in the contents of the filtrate could be obtained. In Table 2 are summarized some of these variations.

TABLE 2.

Behaviour of Various Gelatine Membranes in the Filtration of Black Snake Venom at High Pressures.

Percentage of gelatine.	Time of seasoning in days.	Percentage of thrombin of whole venom in filtrate.	Percentage of other toxic constituents in filtrate.
8%	3	Between 4 and 10%	Not investigated
8·4%	3	Between 1 and 2%	Between 60 and 70%
8·6%	3	Less than 2%	Less than 25%
9·0%	2	Less than 1·0%	About 50%
9·0%	14	Less than 0·7%	Less than 25%
10·0%	3	Less than 0·7%	Less than 10%

A reasonable yield in the filtrate of the toxic constituents of the venom, other than thrombin, was not obtained without the passage of traces of the latter. Most of the thrombin remained in the residue, which, however, contained a considerable amount of gelatine. A few similar results were obtained with tiger snake venom. These experiments showed that this method also was unsuitable for the preparation of large amounts of venom freed from thrombin.

3. ADSORPTION EXPERIMENTS.

The solution of venom was left in contact with a series of insoluble substances previously prepared, and these subsequently removed by means of the centrifuge. The amount of thrombin remaining in the venom solution was estimated by the coagulation *in vitro* of citrated plasma. Determinations *in vitro* were also made of the haemolytic power of the treated venom. A few tests of the toxicity of the venom solution after treatment were made by intravenous injection in rabbits and subcutaneous injection in guinea-pigs.

EXPERIMENT 1. In this experiment the effects on black snake venom of five different adsorbent substances are compared. Four mgm. of the venom dissolved in 4 c.c. of 0·9% sodium chloride and 0·08 grm. of each adsorbent were left in contact for one hour at room temperature, centrifuged, and the solutions placed in a refrigerator overnight. The adsorbents employed were (1) Merck's charcoal, (2) kieselguhr, (3) barium sulphate, (4) barium carbonate, (5) casein. The supernatant fluids were tested by intravenous injection of wild rabbits with 10 certainly lethal intravenous doses. All the rabbits died in 2–3 minutes from intravascular thrombosis, except those injected with venom treated with barium carbonate. These died of respiratory failure from curarization of the diaphragm in 41 and 55 minutes respectively.

Determinations *in vitro* with citrated guinea-pig plasma indicated that supernatant fluid (4) contained less than 0·1 of the thrombin originally in the venom, while haemolysis tests on guinea-pig red corpuscles showed no appreciable loss of haemolytic activity. The other fluids had lost very little thrombin.

Experiment 2. This experiment illustrates the effects of three barium salts and of aluminium phosphate and hydroxide.

The barium salts employed were the oxalate, phosphate, and carbonate. Each substance was used in the proportion of 10^{-4} grm. molecule per c.c. of solution containing 1·0 mg. of black snake venom. The solutions of venom were in contact with them for two hours at laboratory temperature, then centrifuged and stored overnight in a refrigerator. None of the treated venoms showed any loss of haemolytic power towards guinea-pig red corpuscles *in vitro*.

A rabbit weight 1·8 kgs., after receiving 30 certainly lethal doses of the venom treated with barium carbonate, died from respiratory failure, with no trace of intravascular thrombosis. From tests *in vitro* it appeared that the two aluminium salts removed about 50% of the thrombin, barium oxalate very little, barium phosphate 80%, and barium carbonate 94%. It was subsequently determined that, under these conditions, the following compounds did not remove any appreciable quantity of thrombin from black snake venom: calcium phosphate, and the oxalate, carbonate and phosphate of magnesium. Calcium borate and calcium carbonate removed a small proportion.

The following experiments were devised to ascertain the best method of absorbing the thrombin with $BaCO_3$.

Experiment 3. Twenty-five mgs. of venom dissolved in 10 c.c. of 0·9% sodium chloride was treated with 0·0012 grm. molecule of freshly precipitated barium carbonate for 24 hours in a refrigerator, and the solid centrifuged off. A determination *in vitro* with citrated guinea-pig plasma showed that 90% of the thrombin had been removed, while there was no appreciable loss of haemolytic activity.

Before proceeding to large scale preparations a number of experiments were made with tiger snake venom, with a view to finding the best conditions for the adsorption of thrombin by barium carbonate. Freshly precipitated barium carbonate was found much more efficient than commercial barium carbonate under the conditions of Experiment 2.

Experiment 4. This illustrates the effect of variations in the amounts of barium carbonate used and in the concentrations of tiger snake venom employed. The barium carbonate was freshly precipitated with barium chloride by sodium carbonate and well washed. The tiger snake venom was dissolved in water, and the solutions were in contact with the barium carbonate for 18 hours in a refrigerator before being centrifuged. The solutions were diluted with the appropriate sodium chloride solution before testing, so that 1 c.c. was equivalent to 1 mg. of venom dissolved in 0·9% sodium chloride. The results appear in Table 3.

TABLE 3.

Mg. per c.c. of venom.	Weight of $BaCO_3$ in grm. molecules per c.c. of solution.	Percentage of thrombin absorbed.
21·0	0·0001	Less than 50%
10·5	0·0001	50%
5·2	0·0001	60%
2·1	0·0001	98%
1·05	0·0001	94%
1·05	0·00005	98%
1·05	0·00002	60%
1·05	0·00001	50%

EXPERIMENT 5. The effect of varying the pH on the adsorption of tiger snake venom thrombin by barium carbonate. Four lots of 4 c.c., each containing 1 mg. of venom per c.c., were left in contact overnight at 0°C. with 10^{-4} grm. molecule per c.c. of freshly prepared barium carbonate. To the second and third specimens were added respectively 0·1 c.c. and 0·2 c.c. of 0·4N sodium carbonate. The barium carbonate for the fourth specimen was made by passing carbon dioxide into cold saturated baryta solution. The estimations of thrombin were by the coagulation *in vitro* of rabbit and guinea-pig plasmas. The proportions of thrombin remaining in the venom solutions were as follows: (1) 0·1%, (2) 0·2%, (3) 0·3%, (4) 0·05%.

EXPERIMENT 6. The technique established for tiger snake venom was applied to the venom of the black tiger snake. This venom has about twice as much thrombin as that of the common variety. Using a solution of 1 mg. of venom per c.c., and the following amounts of barium carbonate in grm. molecules per c.c., the percentages of thrombin remaining in the venom solution are as follows: 10^{-4} 3%, 6×10^{-5} 6%, 4×10^{-5} 20%, 2×10^{-5} 50%. It is thus more difficult to remove than is that of the tiger snake. The recovery of the neurotoxin is also less complete, only about 50% remaining in the solution in one experiment. Further experiments on these lines showed that for black tiger snake venom it is essential to prepare the barium carbonate in a very finely divided state, by passing carbon dioxide into barium hydroxide dissolved in water below 10°C. The precipitate is collected on the centrifuge and not permitted to dry. It is important that the solution be not alkaline, and advisable to pass carbon dioxide into the chilled solution of venom after suspending the barium carbonate until it is neutral to phenol red. The subsequent readjustment in the cooled solution is sufficiently slow to permit the carbonate to remove the thrombin. At pH 8 much less adsorption appears to occur. Once the thrombin is adsorbed it shows no tendency towards re-solution.

EXPERIMENT 7. The amount of neurotoxin remaining was estimated in three specimens, two of tiger snake venom which had been dried *in vacuo* after treatment with barium carbonate, and one of black tiger snake venom which had not

been dried. The estimation was performed on guinea-pigs by subcutaneous injection, and showed that of the two dried tiger snake specimens one was 100% and the other 90% as active as the dried untreated venom. The proportion recovered in these cases was about 60%. The black tiger snake thrombin-free venom solution was not evaporated, but possessed only about 30% of the neurotoxic activity of the solution before treatment with barium carbonate.

The problem now arose of preparing stocks of a few grms. of each of these venoms, after removal of their blood-coagulating constituents, in order that adequate examination might be made of their physiological effects.

The neurotoxins are too unstable, the quantities of material too small, and the experimental losses too great and costly for any of the recognized methods of treatment to commend itself. Their solutions froth badly, and no "foam-reducer" is permissible, on account of the deleterious action of such substances. It was accordingly decided to attempt to concentrate the dilute venom solutions to dryness *in vacuo* by evaporating from the surface only.

As a preliminary experiment 50 c.c. of a solution of tiger snake neurotoxin in distilled water, prepared as already described, was placed in an open petrie dish over sulphuric acid in a vacuum desiccator. The desiccator was evacuated by means of a mercury vapour pump until the solution froze. The ice evaporated without melting, and the toxin remained in a state of fine division and quite potent. It was found possible to accelerate the drying if desired by means of a small 50 watt electric radiator placed 5 cm. above the surface of the frozen toxin solution. The rapid concentration of larger quantities of dilute neurotoxin solution was carried out as follows: A steel vacuum oven was used, consisting of a cylinder 27 cm. diameter and 45 cm. long, fitted with a door at one end, having a window 10 cm. diameter. It contained the toxin solution, the heater, and the condenser, thus avoiding the retarding effect of a lead tube. The cylinder lay with its axis horizontal. The toxin solution was placed in a whole-plate photographic dish on a stand in the oven. Immediately above it was an electric radiator, consisting of 250 ohms 27 B.S. Nichrome wire, wound in a coiled spiral on the lower side of an asbestos-cement sheet. The current was controlled by an external rheostat. Below the dish was a condenser, consisting of 10 feet of half-inch copper pipe, through which water circulated. Below the condenser was a drainage tray leading to a tube in the floor of the oven to a cooled receiver. The temperature of the liquid was observed on a thermometer placed in it, illuminated by a small electric bulb in the oven. The oven was evacuated by three water pumps. It was found easy after a little experience to maintain a rate of evaporation of about 250 c.c. per hour with very slight loss from splashing. The temperature of the liquid was only 2–3°C. above that of the cooling water. As the evaporation drew to an end the rate of heating was decreased.

Including stops for refilling, etc., about 1,300 c.c. liquid were concentrated to 50 c.c. in 7 hours without much loss of toxin. The precipitate could be washed with distilled water without removing any of the adsorbed thrombin. During the concentration *in vacuo* some barium carbonate settled out of the solution. This was centrifuged off and washed with water. The washings were added to the supernatant fluid, and the whole evaporated to dryness after freezing, as already described.

The final yield amounted to some 50–60% of the neurotoxin originally present, as judged from subcutaneous injection into guinea-pigs. The yield from black tiger snake venom was less, and the proportion of thrombin remaining in the final product, though less than in the early experiments, was greater than in the case of tiger snake venom.

The percentages of nitrogen, as determined by the micro-Kjeldahl method, in two specimens of tiger snake venom that had been treated with barium carbonate were 12·5 and 13·3, while the normal venom from which they were prepared contained 13·1% of nitrogen. As estimated by the subcutaneous injection of guinea-pigs the toxicity of the first specimen was equal to that of the untreated venom, while that of the second was somewhat less. It would thus appear that, while a small proportion of the neurotoxin may be inactivated during the process, there is relatively little contamination of the treated venom with inorganic material from the adsorbent.

The second specimen was examined for thrombin by intravenous injection into rabbits. A dose of 3 mgs. per kg. killed without intravascular thrombosis, while after a dose of 4 mgs. per kg. fibrin was found in the heart of the dead animal. Thus the proportion of thrombin remaining in the venom was between 0·05% and 0·06% of the amount originally present.

The author is greatly indebted to Miss F. E. Williams for the estimation *in vitro* of thrombin and haemolysin; to Miss M. Freeman for determinations of nitrogen in the venoms; and to the Director of the Institute for the *in vivo* estimations of thrombin and neurotoxin.

SUMMARY.

Experiments are recorded on the separation of active constituents of snake venoms by the use of ultra-filtration under high pressures and by adsorption. The latter method was the more effective.

A method is outlined for the rapid evaporation at low temperatures of large volumes of fluids to which distillation *in vacuo* from flasks is inapplicable.

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THE ENVIRONMENTAL FACTORS AFFECTING THE DEVELOPMENT OF THE EGGS OF *SMINTHURUS VIRIDIS* L. (COLLEMBOLA)

by

J. DAVIDSON

(Waite Agricultural Research Institute, University of Adelaide).

(Submitted for publication 2nd December, 1932.)

1. INTRODUCTION.

The most important factors affecting the development and hatching of the eggs of *S. viridis* are temperature and the moisture conditions on the soil surface on which the eggs are laid. These two factors are so intimately interrelated that it is difficult to interpret the influence of the former without reference to the latter.

Within a certain range of temperature the curve which expresses the developmental period of the eggs in relation to temperature is a hyperbola, having the formula $d(t - c) = K$, where d = developmental period in days, t = temperature, and c = developmental zero = ca 5·5°C. (*vide* Davidson, 1931). These conditions hold when the moisture factor is about the optimum. As the moisture conditions depart from the optimum, either in the direction of "too dry" or "too wet," development is retarded, and under extreme conditions may be arrested. The subsequent fate of the eggs under these extreme conditions depends on the stage of development attained and the duration and severity of the adverse conditions.

It has been shown that, under experimental conditions, an atmosphere of not less than 100% relative humidity is necessary for development and hatching of the eggs (Davies, 1930). In the natural state the eggs are surrounded with soil-excreta, and when the moisture content of the surface soil is favourable the necessary moisture conditions are assured. The stability of these conditions depends upon the rainfall-evaporation ratio. When the moisture in the surface soil falls to a low value the humidity in the micro-environment on the soil surface will approximate closely to that of the air above. The relative humidity of the air will fluctuate according to locality, temperature, and other meteorological factors. During a rainless period the moisture available for the eggs from this source will normally be insufficient for their requirements, and development will be retarded or inhibited (Davidson, 1932).

On the other hand, if the surface soil is wet, the eggs become surrounded with a film of moisture, which, probably by restricting the supply of oxygen, retards their development and increases the death rate.

The development curve referred to above was calculated from the emergence of first individuals from a number of egg batches. First emergences include those eggs which are more favourably situated with respect to the environment. The more favourable and uniform the conditions are for all the eggs in a batch, the sooner will the remaining eggs hatch.

The present paper deals with data obtained from batches of eggs maintained under various conditions of temperature and moisture. The eggs used in the experiments were laid on soil having a reaction of pH 6·4. In order to ensure well-formed batches being available, a period of 48 hours was allowed from the time the females were introduced into the oviposition cages. Experience showed that few eggs were laid on the first day, so that the second day was taken as the date the eggs were laid. The number of individuals which hatched in the various experiments were recorded daily, so as to retain the day as a unit. At the end of each experiment the egg-batches were washed through bolting silk and the non-hatched eggs counted. In this way the total number of eggs used in each experiment was known.

The percentage of eggs which hatched varied according to the conditions. In a number of batches, however, even when the conditions were favourable, a few eggs, which showed no outward signs of development, remained at the end of the hatching period. These may have been infertile eggs. It is, however, possible that some eggs may die at an early stage of development before any outward sign of development is visible.[1]

The females will oviposit without mating. Mr. D. C. Swan isolated 40 nymphs, hatched July 27, in separate cages. Ten batches of eggs were laid by 10 females early in September. There was an average of 48 eggs per batch. None of the eggs hatched. The following examples of normal batches, incubated under favourable conditions, illustrate the usual percentage of apparently non-developed eggs which is obtained.

No. of egg batches.	Total eggs.	Eggs which failed to develop.
		%
4	488	5
4	430	7
5	463	3
9	319	5
5	326	3
9	581	2

[1] The first outward sign of development occurs when the eggs have completed about 50% of their development. At this stage the pigmentation of the developing eyes is visible through the chorion. At 20°C. eye spots were visible in certain eggs on the fifth day. The chorion caps were separating on the ninth day, the antennae being visible through the embryonic membrane, and hatching began on the tenth day.

With certain batches 100% of the eggs hatch. It would be necessary to examine in greater detail the eggs which had no outward signs of development, before it could be decided whether they were infertile or eggs which had died at a very early stage of development.

If the conditions surrounding the egg batches are too wet the mortality may be high, especially when the temperature exceeds about 20°C. The batches are more susceptible to attack by fungi and disease under these conditions. Certain of the eggs may become disintegrated, so that it is sometimes impossible to make a reliable count of the number remaining in a batch at the end of the experiment. Normally the two chorion caps separate within about 24 hours of the eclosion of the nymph. If the conditions are too wet, however, they may separate earlier, due to the osmotic tension within the egg. The heavy mortality amongst the eggs when the soil surface is continually saturated is probably partly due to the shortage of oxygen available for the developing embryos, owing to the eggs being covered with a film of water. On the other hand, it would appear that certain eggs can withstand being submerged under water for several weeks (Davidson, 1932b).

Apart from considerations of temperature, the best conditions for development of the eggs in nature occur when the ratio between evaporation and rainfall ensures favourable steady moisture conditions on the surface of the soil. In this respect the soil type and the nature of its vegetative covering is important. In a normal season such conditions obtain at the Waite Institute during May and early June and again in September. During July and August the surface of the soil may be continually saturated, owing to the low ratio of evaporation to rainfall. Therefore, owing to the combination of favourable temperatures and favourable moisture conditions on the soil surface, *S. viridis* is generally more numerous and active during the autumn and early spring months, although it is present in economic numbers throughout the wet season.

2. EXPERIMENTAL DATA ON HATCHING IN RELATION TO TEMPERATURE AND MOISTURE.

With batches of eggs retained *in situ* on the soil it is difficult to maintain experimentally uniform moisture conditions on the soil surface. With favourable temperatures the character of the hatching curve will reflect the influence of the moisture conditions. When the latter are favourable all the viable eggs will hatch soon after the first individuals have emerged. When the moisture factor is unfavourable the hatching period will be considerably extended.

With certain experiments, in order to obtain uniform moisture conditions, the batches were incubated on damp filter paper in petri dishes. With others the eggs were placed singly in rows on the paper, so as to obtain similar conditions for the individual eggs.

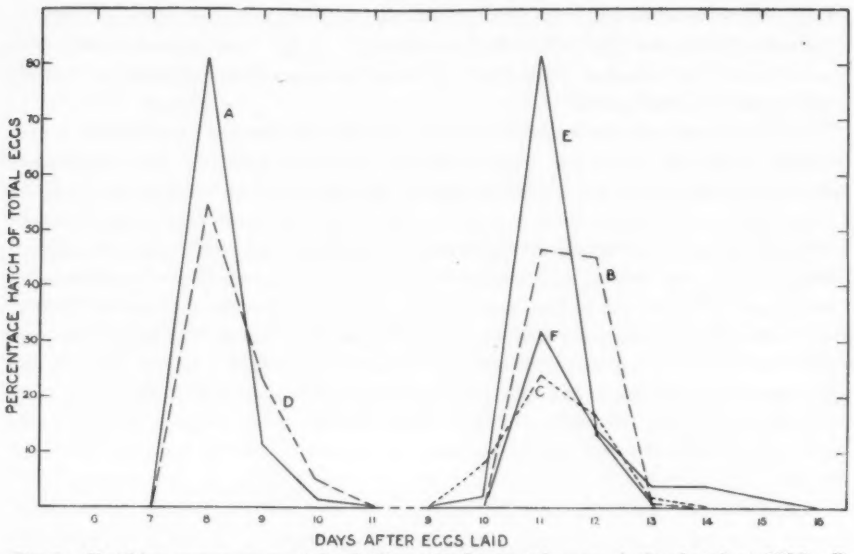

Fig. 1. Hatching curves, eggs of *S. viridis*. (1) Separated eggs: A, incubated at 25°C.; B, 20°C., moisture favourable; C, 20°C., conditions too wet. (2) Complete egg batches: D, 25°C.; E, 20°C., moisture favourable; F, 20°C., conditions too wet.

3. INFLUENCE OF TEMPERATURE, IN RELATION TO MOISTURE, ON THE DEVELOPMENT OF THE EGGS.

Experiment 1. Hatching data at 20°C. and 25°C. with eggs placed singly on damp filter paper in petri dishes.

(a) *Incubated at 25°C., moisture conditions favourable.* (Fig. 1, A.)

Four egg batches; total eggs, 488; 94% hatched; 3 embryos died during development and 25 eggs showed no signs of development by the 11th day.

(b) *Incubated at 20°C., moisture conditions favourable.* (Fig. 1, B.)

Four egg batches; total eggs, 430; 92% hatched; 35 eggs showed no signs of development by the 14th day.

(c) *Incubated at 20°C., on wet blotting paper, moisture conditions being unfavourable (too wet).* (Fig. 1, C.)

Ten egg batches; total eggs, 983; 49% hatched; 353 eggs showed no signs of development by the 15th day; evidently many of them had died at a very early stage of development.

The uniform conditions in Experiments 1(a) and 1(b) are reflected in the hatching curves. Care was taken to ensure that the paper was just suitably damp to produce a uniform moisture round the eggs. Previous experience had shown that if the paper is too wet, poor hatching is obtained, owing to the eggs being covered with a film of water. The effect of this is seen in Experiment 1(*c*), in which a relatively small number of eggs hatched. From one batch only 16 eggs hatched out of a total of 106, which may be partly due to an increased number of infertile eggs. The irregularity in hatching, due to the retarding influence of the excess moisture conditions, in reflected in the hatching curve. (Fig. 1, C.)

Experiment 2. Hatching data at 20°C. and 25°C. from complete egg batches incubated on damp filter paper in petri dishes.

To ensure uniform moisture round the eggs, the egg batches were placed on filter paper resting on moist sand in petri dishes.

(a) *Incubated at 25°C., moisture conditions favourable.* (Fig. 1, D.)

Five batches; total eggs, 463; 85% hatched; 14 eggs showed no signs of development by the 12th day. In one batch a number of non-hatched eggs had made considerable development and doubtless would have hatched later. The average hatch per batch was 80%.

In two instances several individuals died shortly after hatching. They were considerably distended, and death appears to have been due to the saturated condition of the air in the petri dish, which did not permit of a normal regulation of the water balance of the insects. This feature has been observed with adults in a saturated atmosphere. If the insects are kept in atmospheres of low humidity they shrink in size, moisture being lost from the body through the delicate integument. They demand, therefore, a relatively high humidity in the environment. In nature, however, the loss of moisture is partly offset by the water taken in with the food and from free moisture in their surroundings. Under favourable conditions there is an adjustment between the loss of moisture from the body and the water intake by the insect. In a saturated atmosphere, particularly at temperature above about 20°C., when the metabolic processes are proceeding at a high rate, adjustment of this water balance may be seriously interfered with, resulting in death. With lower temperatures the strain on the insect is less severe. In nature the insect is able to move about on the plants, or over the soil surface, and select local situations where the conditions are favourable. For instance, it seeks moistures in the crevices of the soil when the surface is dry. On warm days, when the humidity is high in the crop, the insects may be found feeding on the upper leaves of the plants, where the humidity is lower. On hot, dry days in October adult individuals may be found feeding on the upper leaves of rapidly growing lucerne plants. The water content of the mesophyll tissue on which they feed is evidently sufficient to offset the loss of moisture from the body.

The effect of unfavourable, wet conditions on the development of the eggs at 25°C. is shown in Experiment (b).

(b) *Incubated at 25°C., unfavourable, wet conditions.*

There were five batches. The filter paper was kept wet, so that the soil associated with the batches was saturated, most of the eggs being surrounded with a film of water. By the 7th day, many of the eggs showed the characteristic splitting of the chorion and separation of the polar caps. A total of 23 eggs hatched on the 8th day, two on the 9th. No further eggs hatched. The batches were washed through bolting silk on the 11th day; 27 eggs had apparently made no development. The majority of the remaining eggs had made considerable development, but were dead and partially disintegrated, so that it was impossible to make a reliable count. The batches were badly infected with fungal hyphae.

(e) *Incubated at 20°C., moisture conditions favourable.* (Fig. 1, E.)

Nine batches; total eggs, 319; 95% hatched; 15 eggs showed no signs of development by the 14th day. The favourable conditions are reflected in the hatching curve.

(d) *Incubated at 20°C., unfavourable, wet conditions.* (Fig. 1, F.)

Five batches; total eggs, about 326 (?); about 56% hatched; eight eggs showed no signs of development by the 17th day. About 136 eggs had died at various stages of development. Some were disintegrated so that it was not possible to make a reliable count of the total eggs. The conditions were very wet, most of the eggs being surrounded with a film of water (compare Experiment 2(b)); certain of the newly hatched individuals were unable to escape from the wet film covering the batches and were drowned.

(e) *Incubated at 20°C., moisture conditions favourable.*

Nine batches; total eggs, 581; 89% hatched. Hatching commenced on the 11th day and ceased on the 15th day; 12 eggs remained which showed no signs of development. In one batch 47 eggs were at the eye spot stage and it was not possible to say whether they were dead. In the remaining eight batches the average hatch per batch was 97%.

(f) *Incubated at 20°C.*[2], *moisture conditions variable.*

There were 11 batches, each one placed in separate dishes. The moisture conditions for the individual batches varied somewhat, chiefly owing to the variable size and compactness of the batches, and hatching was irregular. Owing to some of the eggs being partially disintegrated at the end of the experiment, it was not possible to record accurately the total number

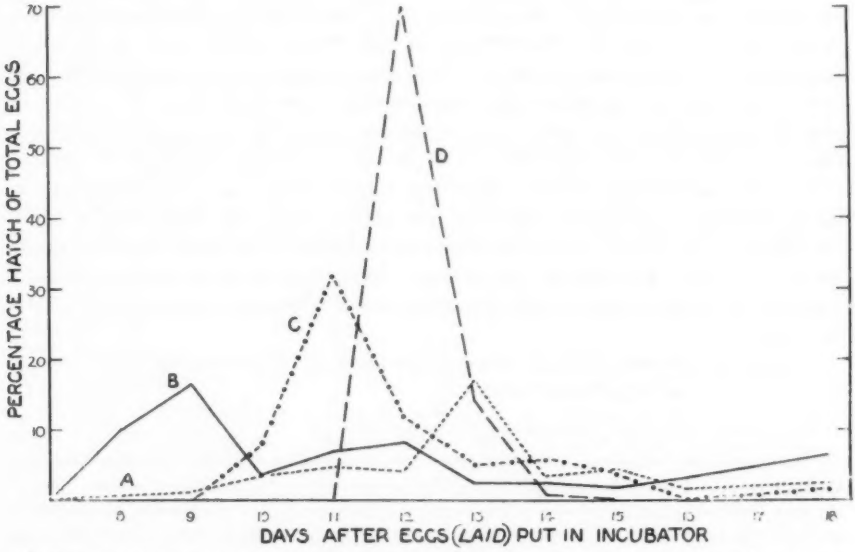

Fig. 2. Hatching curves *S. viridis*, egg batches retained as laid on soil surface: Mean temps. A, 25·7°C.; B, 23·7°C.; C, 21°C.; D, 19°C.

[2] The electric current was shut off for three days, during which the temperature dropped to about 16°C.

of eggs; it was about 1,180. A few individuals hatched on the 11th day, and 638 during the 11th to the 15th day. The experiment was terminated on the 18th day, when a total of 903 eggs had hatched=ca 76%. A relatively large number of eggs, which showed no signs of development, remained in three batches, from which only 11, 2 and 5 eggs hatched. It is improbable that these batches consisted of such a large proportion of infertile eggs, and are considered to include thoses which died at an early stage of development.

Experiment 3. Hatching data from egg-batches retained *in situ*, as laid on the soil, and incubated at different temperatures with various conditions of soil moisture.

The eggs were retained on soil contained in 2½ in. flower pots, one batch to each pot. The pots were placed in the compartments A to E of a multiple temperature incubator. They were embedded in moist sand in order to prevent rapid loss of moisture from the soil, three successive series of experiments being made.

With a modification of the technique improved moisture conditions on the surface of the soil were obtained in the successive series. This is reflected in the character of the hatching curves (figs. 2-4).

A. *Series I.* Compartments A-D (fig. 2).

Compt.	Temp. °C. Range.	Mean T.	Total eggs.	Total eggs hatched %	Peak day of hatching.
A	25–26	25·7	191	42	13
B	23–25	23·7	171	61	9
C	20–22·5	21	145	67	11
D	18–20	19	198	85	12

The moisture conditions on the soil surface were unfavourable, being too wet; the conditions were better in D, and less severe at this lower temperature. This is reflected in the character of the hatching curves.

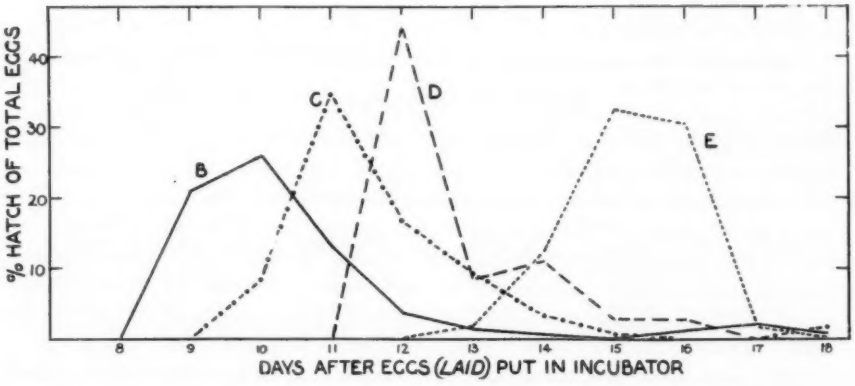

Fig. 3. Hatching curves *S. viridis*, egg batches retained as laid on soil surface: Mean temps. B, 23°C.; C, 20·6°C.; D, 18·6°C.; E, 15·4°C.

B. *Series II.* Compartments B-E (fig. 3).

Compt.	Temp. °C. Range.	Mean T.	Total eggs.	Total eggs hatched %	Peak day of hatching.
B	22–24	23	266	68	10
C	19–22	20·6	239	74·5	11
D	18–19·5	18·6	147	70	12
E	15–16·5	15·4	275[3]	79	15

The moisture conditions on the surface of the soil were more favourable in this series, which is reflected in the hatching curves. These curves show the delayed hatching of many eggs, which is associated with the variable conditions due to the form of the batches (compare fig. 1, A and E).

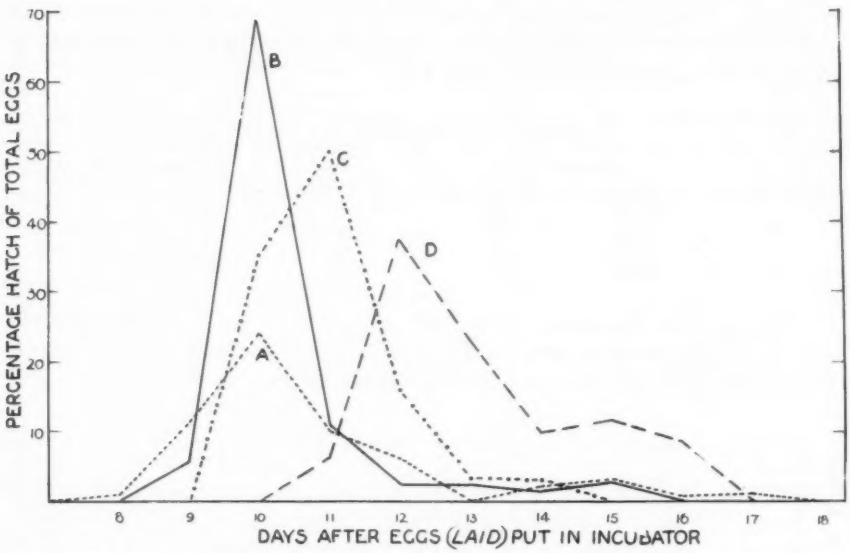

Fig. 4. Hatching curves *S. viridis*, egg batches retained as laid on soil surface: mean temps. A, 25·5°C.; B, 23°C.; C, 20·8°C.; D, 19°C.

C. *Series III.* Incubated in compartments A-D (fig. 4).

Compt.	Temp. °C. Range.	Mean T.	Total eggs.	Total eggs hatched %	Peak day of hatching.
A	24·5–26·5	25·5	197	58	10
B	22–24	23	263	93·5	10
C	20–22	20·8	277	99	11
D	18–20	19	199	97	12

The hatching curves show that the moisture conditions were more favourable than in Series I. From the results obtained in compartment A in both series it

[3] The eggs were in Compt. A for 6 hours at 24°C. on first day.

is evident that at about 25°C. the moisture conditions must be extremely favourable in order to obtain good hatching (compare fig. 1, A).

4. INFLUENCE OF DRY CONDITIONS ON THE DEVELOPMENT OF THE EGGS.

It has been shown in an earlier paper (Davidson, 1932) that development of the eggs is retarded when the moisture content of the surface soil falls below a certain value, and may be completely arrested if low values are maintained. These moisture values cannot be sharply defined; they depend upon many considerations, of which the soil type and the temperature and relative humidity of the air are the most important. In a soil which is suitably moist the eggs are adequately supplied with moisture, owing to the water associated with the soil particles being absorbed into the soil-excreta covering the eggs. During a rainless period, when the soil is drying out, this source of moisture fails when particular moisture values have been reached. With moderate temperatures and a high relative humidity in the air, ample moisture may be retained by the soil for some time, depending chiefly on the soil type and its vegetative covering.

Field observations at the Waite Institute show that the moisture conditions for general development and hatching are most favourable when the moisture value of the surface soil is about 75% saturation. As the moisture falls to a value of 40% saturation a retardation of development occurs, and there is a marked decrease in the number of individuals which hatch. These features become accentuated when the value falls below 40% saturation. Above 80% saturation the conditions tend to become too wet, resulting in retardation of certain eggs and a decrease in the number which hatch. These conditions are accentuated as the moisture value approaches saturation and free water occurs on the soil surface.

When the moisture surrounding the soil particles composing the surface soil has evaporated the humidity of the air surrounding the eggs may fall below the value necessary for their devolpment. During a rainless period the air on the soil surface may be saturated temporarily, in local situations (dew, fog), and development of the egg, and even hatching, may occur at lower soil moisture values than those referred to above; but suitable moisture conditions on the soil surface, over long periods, depend upon a favourable ratio of rainfall to evaporation.

When the soil is drying out some eggs continue development, for a time, after moisture values in the surface soil have fallen below 40% saturation. This is partly due to the moisture available for particular eggs in localized situations, and also to the moisture available within the egg (Davidson, J., 1932).

It appears that the degree of moisture necessary for general hatching to take place is higher than that required for development of the egg to continue. Consequently, when the soil is drying out, many eggs continue development after general hatching has ceased. This causes a "banking up" of the eggs at a rela-

tively advanced stage of development. Therefore, when the soil again becomes suitably remoistened, general hatching takes place after a relatively short period. The proportion of the eggs which survive the dry period chiefly depends on the severity and duration of the conditions. The importance of this feature in nature is evident, in that it enables the species to take full advantage of temporary periods of favourable moisture conditions.

The following experiments illustrate the features referred to above, and confirm the observations described in a previous paper (Davidson, J., 1932).

Experiment 4. Hatching data from egg batches incubated for 8, 6, 4, 2, 0 days at 21·5°C., then dried for 7 days at 22°C., and 49% R.H., afterwards remoistened at 25°C.

This experiment included seven series of egg batches each consisting of 12 batches (Series A-G). The batches were laid on soil in pots, then transferred to damp filter paper in petri dishes. The experiment was arranged so that with Series A to F the eggs were incubated for 10, 8, 6, 4, 2, 0 days respectively at 21·5°C. The developmental period at this temperature is about 10 days, so that the approximate amount of development was known. Hatching actually commenced with Series A on the 10th day, and this series was then discarded. Series B-F were then placed in an incubator at 22°C. in an atmosphere of 49% R.H. and the batches allowed to dry out for 7 days. They were then remoistened and placed in an incubator at 25°C., the hatching data being recorded daily. With Series G, the batches were not dried, being retained suitably moist throughout at 25°C. The number of non-hatched eggs in the different series was recorded at the end of the experiment, the data from which is given below.

Series.	Days at 21·5°C. before drying.	Day hatching commenced at 25°C.	Total eggs.	Total hatch.	% hatch.
A	10	Hatching commenced on tenth day.			
B	8	2	479	411	86
C	6	2	375	349	93
D	4	3	231	104	45
E	2	3	227	195	86
F	0	—	599	0	0
G	not dried	8[4]	321	225	70

The hatching curves are shown in fig 5. It is clear that many eggs continued development for a time during the drying period. When remoistened at 25°C. they hatched out much earlier than would be expected had they not done so. A high percentage of the eggs hatched, showing that the drying conditions were not severe. With Series F, however, none hatched. These features have been discussed in an earlier paper (Davidson, J., 1932).

The amount of development attained during the drying period can be calculated. The eggs in Series B–E had completed respectively 8/10ths, 6/10ths, 4/10ths, and 2/10ths of their development when placed in the dry environment at 22°C. If development had ceased immediately, the time required to complete their development, when remoistened at 25°C. would be ca. 1·5, 3, 4·5, and 6 days respectively. Hatching actually commenced after 2, 2, 3, and 4 days in the respective series. Development had continued to a certain extent during the

[4] The eggs were at 22°C. on the first day.

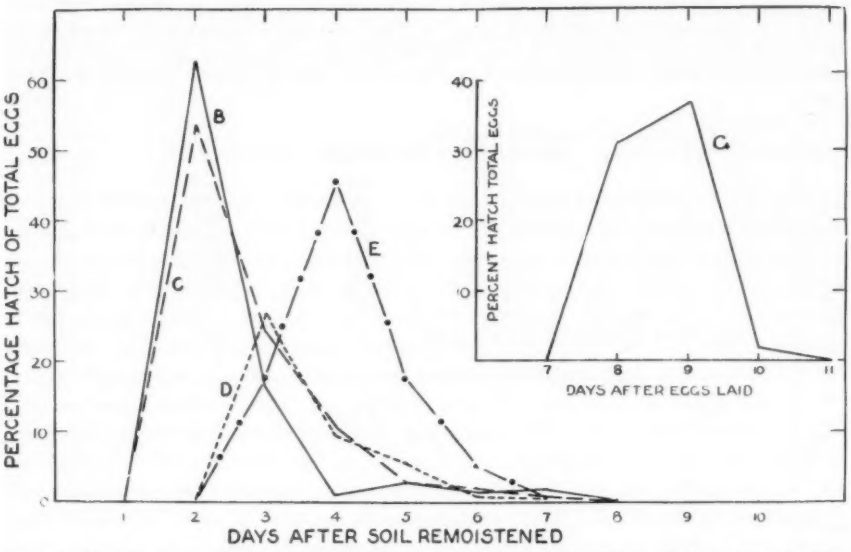

Fig. 5. Hatching curves *S. viridis*, egg batches incubated at 21·5°C. for 8 days (B); 6, (C); 4, (D); 2, (E) respectively, then dried for 7 days at 22°C., afterwards re-moistened and incubated at 25°C. (G), incubated at 25°C., not dried.

drying period, being finally arrested owing to the absence of the necessary moisture. The amount of development is greater in Series D and E, in which the eggs were in a less advanced stage of development at the commencement of the drying period. This may be due to the greater moisture requirements of the older embryos.

This continuation of development when the soil is drying out results in a "banking up" of many eggs at a fairly advanced stage of development. It also shows that the moisture requirements for completion of development and general hatching are greater, since no eggs hatched in the series B to F during the drying period.

The "banking up" of the eggs at a relatively advanced stage of development is shown in the hatching curves in fig. 6, since a large percentage hatched within a comparatively short time after batches were remoistened. The character of the hatching curve is affected by the stage of development attained by the eggs when the dry conditions set in, and by the degree of mortality as affected by the duration and severity of those conditions. The feature discussed above are seen again in Experiment 5.

Experiment 5. Hatching data from egg batches incubated for 7 to 0 days at 25°C., then dried for 35 days, afterwards remoistened at 22°C.

There were eight series of egg-batches (Series A-H), which were so arranged that they had 7 to 0 days incubation respectively at 25°C. They were then left exposed to dry out in the laboratory for 35 days, afterwards being remoistened and incubated at 22°C. In a further series (K), the batches were retained throughout at 25°C. and not dried. The data are given below.

Series.	Days at 25°C. before drying.	Day hatching commenced at 22°C.	Total eggs.	Total hatch.	% hatch.
A	7	3	412	25	0·5
B	6	2	242	2	0·8
C	5	2	404	109	27
D	4	2	405	345	86
E	3	2	410	230	57
F	2	2	601	441	73
G	1	2	368	223	67
H	0	4	332	2	0·6
K	Controls retained at 25°C. not dried		361	203	56

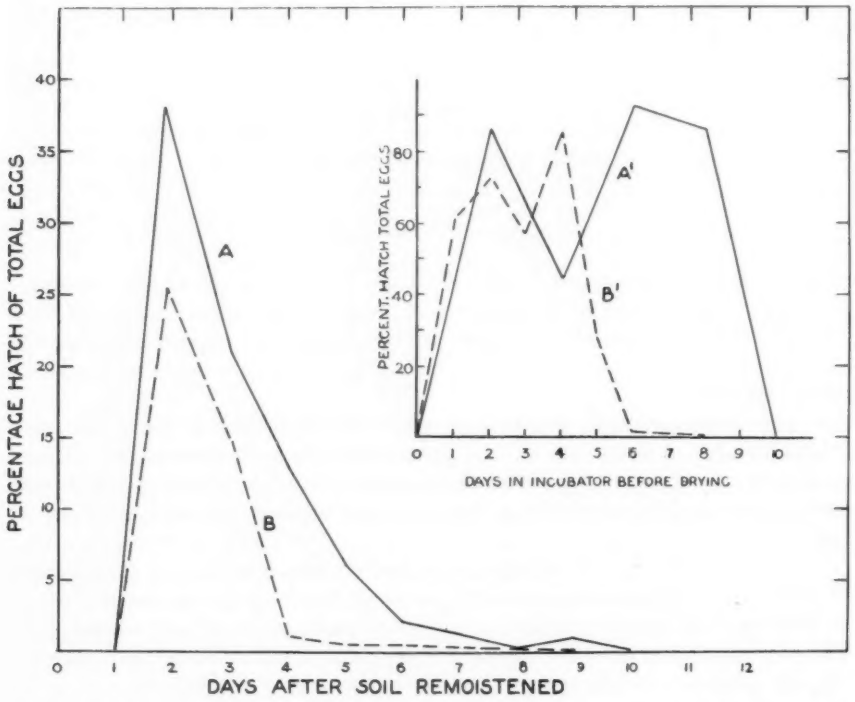

Fig. 6. Hatching curves *S. viridis*, egg batches incubated for varying periods, then dried, afterwards re-moistened. A, percentage daily hatch of total eggs from Series B to E (Expt. 4); B, Series A–H (Expt. 5); A', B', percentage hatch plotted against number of days in incubator before eggs were dried: A', Series B to E; B', Series A to H.

[5] Five individuals hatched on first day of drying period; these are not included.

The daily hatch from all the series is shown in fig 6B; in 6B[1] the number of eggs which hatched per batch is plotted against the number of days at 25°C. before drying. The results obtained confirm those obtained in previous experiments (*vide* Davidson, 1932).

GENERAL CONCLUSIONS.

The temperature and moisture in the micro-environment on the soil surface on which the eggs of *S. viridis* are laid are the most important factors which influence their development and eclosion. The influence of temperature is intimately associated with the moisture conditions. If dry conditions obtain, development of the eggs is retarded, and may be completely arrested. Holdaway (1927) showed that eggs retained in a dry environment remained viable for 271 days. At the end of that period the eggs were remoistened, and began to hatch after 12 days. This author considered that the undeveloped egg was the only stage capable of resisting adverse dry conditions, but the eggs in this experiment had evidently undergone some development prior to being remoistened. Certain partially developed eggs offer considerable resistance to adverse dry conditions. The insects normally will lay only on moist soil, and if the soil is drying out the eggs will develop, for a time, if temperature conditions are favourable. The resistance of partially developed eggs to temporary dry periods is therefore a useful safeguard for the species. The results from experiments previously described (Davidson, 1932) show that eggs which have made only slight development and those which are approaching the final stages are less able to withstand subsequent dry conditions. The chorion is very delicate at oviposition.

It has been shown that partially developed eggs are able to retain their vitality when maintained at temperatures below the developmental zero (Maclagan, 1932).

If the moisture conditions on the surface of the soil are too wet, such that the eggs become covered with a film of water, development of the eggs is retarded and may be inhibited. This feature has been noted by Maclagan (1932). On the other hand, observations show that certain partially developed eggs may be completely submerged for several weeks and still retain their viability.

Although an over-saturated condition of the surface soil is unfavourable for the eggs, relatively moist conditions are required for general hatching to take place. With the Waite Institute soil the moisture conditions on the soil surface are more favourable for general hatching at values about 75% saturation. Owing to evaporation of the moisture surrounding the soil particles, the atmosphere on the soil surface may be humid for a time after a dry period sets in. The duration of these conditions will depend upon the power of the soil to retain moisture, its vegetative covering, and the evaporating power of the air. Moisture is absorbed by the soil-excreta covering the eggs from the surrounding soil particles with

which they are in contact. When the soil is drying out the available moisture may fall too low to permit of general hatching, but there will be sufficient in the soil-excreta and in the eggs themselves to enable certain of the eggs to continue development for a time after this low moisture value has been reached. As the dry conditions continue these sources of moisture fail, and development is inhibited. The fate of the eggs depends upon the stage of development attained and the duration and severity of the drought period.

With favourable moisture the development of the eggs in relation to temperature may be expressed as a hyperbolic curve having the formula $d(t - c) = K$, where c = developmental zero = ca. 5·5°C. The straight line limits of the velocity curve lie between about 9°C. and 25°C., provided that the moisture conditions are about the optimum.[6]

Maclagan (1932) gives the approximate straight line limits of development for the species in England as 9°C. to 21°C. The data on which these values are based are not given. This author states that hatching occurs in 19 days at about 14°C. to 16°C. It will be seen from the development curve referred to above that the incubation period for the species in South Australia at 14°C. is 19 days, at 15°C. 17 days, and at 16°C. 15 days. Temperatures about 20°C. and over are harmful to the developing eggs if the moisture conditions are on the wet side of the optimum. With favourable moisture, however, 94% of a total of 488 eggs hatched at 25°C., and 92% of a total of 430 eggs at 20°C. With similar temperatures, in a dry environment, development is arrested, and certain of the partially developed eggs remain viable during temporary dry periods. In regions of summer rainfall, resulting in frequent moisture changes on the soil surface, accompanied by high temperatures, there will be heavy mortality amongst the developing eggs during the summer period. The best conditions for development and general hatching will occur during the periods in which there is a favourable ratio between the mean daily rainfall and evaporation, accompanied by moderate temperature. Such conditions occur, in normal seasons, at the Waite Institute during late April to the end of June, and again in September. During these periods the insect is more active and numerous, although it also occurs in economic numbers throughout the winter months.

Mating is not essential for oviposition to take place, and unmated females apparently lay infertile eggs. With egg batches laid in captivity certain eggs fail to develop, and are presumed to be infertile.

SUMMARY.

1. Temperature and moisture conditions on the soil surface are the two important factors affecting the development and hatching of the eggs of *S. viridis*.

[6] Hatching data was not obtained below 9°C., owing chiefly to the difficulty of maintaining suitable moisture conditions on the soil surface during the long incubation period required at these low temperatures.

2. The results of experiments with eggs incubated under different moisture conditions are presented.

3. When the moisture conditions depart from the optimum, either in the direction of "too wet" or "too dry," development is retarded and hatching becomes more irregular; with wet conditions, particularly at temperatures of 20°C. or over, the death rate is high; with favourable moisture conditions at 25°C. 94% of a total of 488 eggs hatched, and at 20°C. 92% of a total of 430 eggs hatched.

4. The best moisture conditions for development and hatching in nature obtain when there is a favourable ratio between rainfall and evaporation; at the Waite Institute the most favourable conditions for general hatching obtain when the moisture content of the surface soil is 60–80% saturation. (The saturation value of the soil on a wet soil basis is 24–25% moisture.)

5. When the soil is drying out development and hatching of the eggs continues when the soil moisture falls below this value; below 50% saturation development is considerably retarded and hatching is greatly reduced; with soil moisture values below 40% comparatively few eggs hatch; the effects of these conditions, however, vary somewhat according to the temperature and relative humidity of the air.

6. When the soil surface is saturated development of many eggs is retarded, and there is a marked increase in the death rate.

7. There is a close relationship between the effect of these adverse conditions and the prevailing temperature; they are more severe at temperatures above about 20°C. and at low temperatures, owing to the slow rate of development.

8. When the soil dries out development and hatching is inhibited, but certain partially developed eggs retain their viability during the drought period.

9. The straight line limits of the velocity curve of development of the eggs lies between 9°C. and 25°C., provided moisture conditions are about the optimum.

10. Unmated females lay apparently normal batches of eggs; from ten unmated females ten batches were obtained, containing an average of 48 eggs; none of the eggs hatched, and were presumably infertile.

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THE INTAKE OF IONS BY THE PLANT AND ITS RELATION TO THE RESPIRATION OF THE ROOT

by

ARTHUR H. K. PETRIE

(Waite Agricultural Research Institute, University of Adelaide).

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INTRODUCTION.

In this paper an attempt is made to present a working hypothesis for some of the processes concerned in the intake and accumulation of mineral ions by the higher plant. Many of the data upon which such a hypothesis is based are most conveniently obtained from the study of isolated tissues or of lower organisms possessing very large cells. Investigation of such systems, however, requires to be supplemented by observations on the whole plant, and in the present work some observations of this kind are recorded, and shown to conform with expectation in terms of the working hypothesis.

The writer desires to express indebtedness to Dr. S. W. Pennycuick, of this University, for his kindness in reading the manuscript of the paper.

DEVELOPMENT OF WORKING HYPOTHESIS FOR THE PROCESS OF ION INTAKE.

Intake of Ions by a Single Cell.

The elaborate nature of the physiological system of the cryptogamic plant makes it desirable to consider firstly the intake of ions by a single typical plant cell; the conclusions reached may then be applied to the more complex system of the plant as a whole. In such a cell we visualize at least four possible mechanisms causing absorption of ions from the environment.

The first of these is chemical fixation, consisting of synthetic reactions, wherein $Mg^{\bullet\bullet}$, NO_3', SO_4'', H_2PO_4', and other ions are transformed into constituent parts of various organic compounds. Such syntheses lead to growth and multiplication, and as long as these processes continue ions will pass into the cell.

The second mechanism depends on the existence in the cytoplasm of indiffusible colloidal ions, such as those of the proteins. If in any phase of the cytoplasm indiffusible anions are in excess of indiffusible cations, then in this

phase diffusible cations will proceed to an equilibrium at which their concentrations are higher than in the external medium: the reverse will occur if indiffusible cations are in excess. The consequences of this Donnan system have been discussed elsewhere (Briggs and Petrie, 1928).

The third mechanism is that by which free ions are accumulated in the vacuolar sap, where colloidal ions are apparently absent. The concentrations of most ions in the sap are greater than in the external solution; this has been best shown for large-celled algae, such as *Valonia* (Hoagland and Davis, 1928), *Chara* (Collander, 1930), and *Nitella* (Osterhout, 1923), from which the sap can be obtained uncontaminated. In the higher plant it is not possible to obtain the vacuolar sap in its natural condition, although fluid can be expressed which probably gives an indication of the composition of the real sap. Analysis of such expressed fluid shows again comparatively high concentrations of unfixed ions in the cells (Teakle, 1929; Hoagland, 1919). We have considerably less knowledge of the ions present in the protoplasm.

Accumulation until a higher concentration of ions is reached in the vacuole than in the external solution necessitates the performance of work, and hence a source of energy must be present. This energy, like most of that transformed in the vital activity of the organism, is probably rendered available by respiratory oxidation.[1] It is possible that respiratory energy may similarly be utilized in the accumulation of ions in some of the phases of the cytoplasm.

The fourth mechanism is that of adsorption on the protoplasmic colloids. We may expect the amounts of ions so held to be in definite relation to the concentrations of free ions in the aqueous phases of the protoplasm.

We do not know whether the concentrations of ions in the cell-sap increase indefinitely, or whether accumulation ceases when the concentrations have attained a certain value. During the growth of the cell, however, the amount of free ions is being continuously depleted by chemical fixation; even when fully grown some cells may continue to supply to others ions or metabolites containing inorganic elements. Thus accumulation may continue for a considerable period, even though the increase in concentration may have ceased, so that a continuous supply of energy would be necessary for the maintenance of the internal concentrations. Moreover, the rate of accumulation will be dependent on the rate of supply of energy, which may depend on the rate of respiration;[2] if therefore fixation or removal of ions—processes dependent on the ionic concentrations—

[1] The mechanisms whereby the energy rendered available in this process is converted into the various forms in which it manifests itself in the activity of the cell are very imperfectly understood. A hypothesis of Briggs (1930) suggests a possible mechanism in which the free energy necessary for ion accumulation in the vacuole is made available by alternate transference across the cytoplasmic membrane of H- and HCO_3-ions.

[2] Such a relationship is not inevitable: within limits a change in the rate of respiration may conceivably not change the rate of all the processes depending on respiration for energy supply; it depends on the manner in which the various oxidative mechanisms of the cell are interlinked.

is sufficiently rapid, the rate of respiration may limit the maximum concentration attained in the cell-sap. It should be pointed out, however, that when once a certain ionic concentration is attained, provided ions are not removed from the sap by fixation or translocation, a continuously operating force is sufficient to maintain such concentration, and further energy is not necessarily required; but it is possible that in the living cell this force is continuously being dissipated, so that a continuous supply of energy is necessary for its maintenance. This presents the possibility of the magnitude of the force maintained, and hence the maximum concentration of ions attainable, being a function of the rate of respiration, even in the absence of removal of ions by fixation or translocation. A dynamic equilibrium would thus be set up.

The Donnan system and adsorption will set definite ionic equilibria, but with the growth of the cell they will increase in capacity, and additional ions will enter, tending to maintain the equilibria within. In addition the intensity of all the intracellular processes may vary with the physiological state. There may be changes in the concentrations of indiffusible ions, or in the rate of respiration; these quantities may be altered in magnitude by a change in the internal or external hydrogen-ion concentration (cf. Briggs and Petrie, 1928; Thomas, 1931). Such alterations may cause readjustments by inward or outward movement of ions. While an increase in the rate of respiration may cause an increase in concentration of ions of both charges in the vacuole, it may, by increasing the hydrogen-ion concentration of the cytoplasm, result in an increase of diffusible anions, but a decrease of diffusible cations in the phases containing indiffusible ions. These possibilities are considered more fully in other papers (Briggs and Petrie, 1928, 1931). The equilibria are also most probably dependent upon the external ionic concentrations, which may vary.[3] Finally, changes in the amount of adsorbed ions may be produced by changes in the colloidal organization of the protoplasm.

The permeability of the plasma-membrane may also play a part in this system. Complete impermeability to any ion will cause unequal distribution of the diffusible ions on the two sides of the cell-membranes by setting up Donnan equilibria. A low degree of permeability may temporarily produce the same effect; if also it limits the rate of chemical fixation of the slowly penetrating ion it will prevent the ion from reaching the equilibria set by the internal mechanisms[4]; this will enhance and prolong the Donnan effect; if the slowly penetrating ion, however, ultimately reaches equilibrium concentration within the cells, such permeability phenomena will have no effect on the final equilibria attained by any of the ions.

The operation of these mechanisms is suggested as a working hypothesis to

[3] For a discussion of these ionic equilibria see the papers by Briggs (1930, 1932).

[4] Attainment of equilibrium will also be prevented if the rate of entry of the ion is insufficient to keep pace with the growth of the cell.

explain some of the phenomena of ion intake by a typical plant cell. In the next section we shall consider how this hypothesis may be applied to the system of many different kinds of cells massed into the complex morphological structure of the plant as a whole.

Intake of Ions by the Whole Plant.

The first stage in the entry of ions into the plant is their passage into the root system. We may consider each cell in the cortical region of the root through which the transpiration stream passes as of the type of our hypothetical cell. Chemical fixation in this region, however, is probably slight, as the cortical tissue of the absorbing zone is not engaged in active synthesis. The amount of ions in the cells of the cortical region is nevertheless depleted by transference into the xylem; little is known of the mechanism of this transfer; the removal of ions from the root, however, will be followed by further intake from the environment caused by the intracellular processes we have already discussed.

It is not expected that there is any relation between the simultaneous processes of water intake and ion intake by the root system,[5] except in so far as the movement of water may break up diffusion shells. A change in the water content of the cells will of course alter the concentration of the ions inside and hence tend to produce readjustments. When we pass to the second stage of the system, however, that of the vascular tissue of the stem, the ions must be borne upwards by the ascending water stream. There is much evidence that the rate of ion intake by the plant and the rate of transpiration are unrelated (e.g., Muenscher, 1922); this suggests that the rate of entry of ions into the base of the conducting column is very slow compared with the normal rate at which they can be removed by the ascending water column.[6]

At the top of the conducting column the ions arrive at the leaf, where some of them undergo continuous chemical fixation in metabolism, and some continue their movement beyond the leaf, passing down the phloem to other sinks. These processes will cause a constant drain on the supplies brought by the ascending sap. In addition to this, the leaf cells will have free and adsorbed ions, in amounts determined by the physiological state of the cells, the concentrations of ions present in the ascending water stream, and the rate at which the ions are able to penetrate the cells.

The Relation of Ion Intake to the Respiration of the Root.

According to the hypothesis developed in the foregoing pages, the supplies of ions for the plant are drawn from the free ions in the absorbing region of the

[5] If at any time the rate of water intake were significantly governed by the phenomenon known as root pressure, and if this phenomenon is the result of energy expenditure by the root cells, then, if the different forms of energy expenditure of the cell vary together in magnitude, there would be a relationship between the processes of ion intake and water intake.

[6] See discussion in Mason and Maskell (1931).

root. The root thus represents a concentration-mechanism interpolated between the ultimate source of ions, the very dilute external medium, and the ultimate sink, the aerial shoot and other growing parts of the plant. The rate at which ions may be taken into the growing shoot may therefore depend within limits on the rate at which they can be supplied by the root, which may be limited in turn by the concentrations of free ions in the living cells of the absorbing region. Since these cells are highly vacuolated one can expect the greater part of the free ions to be in the cell-sap; and we have suggested that the concentrations of free ions attained in the cell-sap may be dependent upon the rate of respiration of the cells; it is therefore possible, provided other factors are not limiting, that the rate of supply of ions to the shoots is similarly dependent upon the rate of respiration of the absorbing cells of the root. The possibility of such a relationship is of considerable practical importance, for the root-system, on account of its normally subterranean environment, is likely to have its rate of respiration influenced by ecological factors.

Some data have recently been obtained by the writer which are of interest in considering the possibility of this relationship; they concern a study of the effect of artificial alteration in the rate of root respiration on the intake of ions by the plant. These data are presented in the following section.

EXPERIMENTAL.

The experiments considered in this work were carried out upon a pure line of Algerian oats, plants of which had been grown in water-culture[7] until they had reached the age of five weeks. Adventitious roots were cut off as produced, so that the whole process of absorption was performed by the primary roots. Plants as uniform as possible were placed in groups of three, with their root-systems in bottles containing 600 c.c. of culture solution of the same composition as that in which they were initially placed after germination. An air-tight seal was carefully made with grafting wax at the point of insertion of the plant through the cork, viz., at the junction of root and stem. A moisture-laden gas stream was then bubbled through the culture solution in each bottle, and thence through a Pettenkofer tube, in which baryta was placed to obtain a measure of the rate of evolution of carbon dioxide by the root-system. Actual determinations of respiration rate were made at intervals throughout the course of the experiment; care was taken to test the seals before and after each determination, as these were frequently broken by the growth of adventitious roots.

In each experiment the bottles were divided into two sets, through one of which was bubbled ordinary air freed from carbon dioxide, and through the other nitrogen containing approximately 5% or 2–3% oxygen. It will be seen

[7] The composition of this solution was as follows in gm. per litre: KNO_3, ·3; $Ca(NO_3)_2$, ·8; KH_2PO_4, ·3; $MgSO_4.2H_2O$, ·3; KCl, ·2; $FeCl_3.6H_2O$, ·025; $MnSO_4.4H_2O$, ·00064; NaCl, ·1.

from the tables of results that the low oxygen treatment produced an obviously significant decrease in respiration rate. Five or six replicates were used for each treatment. The water removed from the culture solution by the plant and by the gas-stream was replaced at intervals by distilled water. The dry weights of the shoots and roots of each replicate were determined at the end of the experiment, and an estimate of the initial values of these quantities was made on a similar set of replicates at the beginning of the experiment.

The ions removed by the plants from each solution were determined by analysis of the initial and residual solutions.[8] The results of three typical experiments are given in Tables 1 to 3.

TABLE 1.

(Duration of experiment 12 days.)

Treatment.*	Resp.†	Final D.W. (gm.)		Mean. D.W.	Ion Uptake in gm.-ions × 10^{-5}/gm. D.W. Rts.				
		Rts.	Shts.	Rts. (gm.).	$K^{\cdot}$	$Ca^{\cdot\cdot}$	$PO_4^{\prime\prime\prime}$‡	$SO_4^{\prime\prime}$	$NO_3^{\prime}$
21% O_2	7·83±2·49	0·3694	1·0549	0·2283	603	262	226	54·7	2329
„	9·93±2·54	0·2817	0·9321	0·1845	657	260	251	55·8	1975
„	6·59±2·01	0·3145	0·8761	0·2009	573	280	256	60·7	2182
„	5·80±1·89	0·3252	0·9508	0·2067	540	272	245	62·4	2421
„	5·50±1·93	0·2984	0·9464	0·1928	684	301	278	68·9	2831
Mean	**7·13**	**0·318**	**0·952**	**0·203**	**611**	**275**	**251**	**60·5**	**2346**
S.D.	—	±0·033	±0·065	—	±59	±16	±19	±5·7	±318
5% O_2	1·84±0·63	0·1783	0·5384	0·1327	567	209	87	61·0	2365
„	1·63±0·60	0·0705	0·6480	0·0705	354	69	84	12·8	516
„	1·77±0·52	0·1254	0·5668	0·1068	549	27	60	58·1	1715
„	2·27±0·54	0·1145	0·5670	0·1009	153	25	47	32·7	1089
„	1·55±0·53	0·2473	0·9286	0·1672	585	170	156	53·3	2016
Mean	**1·81**	**0·147**	**0·650**	**0·116**	**442**	**100**	**87**	**43·6**	**1540**
S.D.	—	±0·068	±0·161	—	±186	±85	±42	±20·5	±740
P	—	—	—	—	8%	1%	<1%	11%	4%

Initial dry weight roots = 0·087 ± 0·008 gm.
„ „ „ shoots = 0·203 ± 0·012 gm.

* In this and subsequent tables the rows of figures correspond to separate replicates, each consisting of three plants in 600 c.c. culture solution.

† In mgm. CO_2/gm.-hr. Mean and standard deviation of five observations varying in length from 4 to 24 hours. The dry weight of the roots (D.W. Rts.) at each determination was based on the assumption of a linear increase from the estimated initial to the observed final weight. The second replicate of the 5% O_2 treatment was an exception where it was assumed that the dry weight of the roots remained constant at 0·0705 gm.

‡ Expressed for convenience as $PO_4^{\prime\prime\prime}$ ions; actually the ions taken in will be mainly $H_2PO_4^{\prime}$ and $HPO_4^{\prime\prime}$.

8 Potassium was determined as perchlorate; calcium by a modification by Mr. R. E. Shapter of this Institute of the method of McCrudden (1911-12); sulphate as barium sulphate; chlorine by an electrometric method devised by Mr. R. J. Best (1929) of this Institute; phosphate by the method of Zinzadzé (1931), and nitrate by the phenoldisulphonic acid method. All results expressed are the means of duplicates and are liable to a maximum error of 2–3% in the case of phosphate and nitrate, and 1% in the other determinations. The thanks of the author are due to Messrs. C. S. Piper and R. E. Shapter for valuable advice on analytical methods.

DISCUSSION.

Experiments of the type described in the foregoing section yield data for the intake of ions by the whole plant at two different respiration rates of the root-system. Were all other attributes of the plants constant, we could state at once the change in the rate of ion intake associated with reduced root respiration. The results given in Table 1, however, show that the position is complicated by the fact that the growth rate of the plants was influenced by the treatment: growth of the roots, and to a less extent that of the shoots, has been inhibited by reducing the oxygen supply to the former. This might be expected on several grounds: diminished rate of respiration will reduce the amount of energy available for growth processes in the root; diminished mass of roots alone, apart from the possibility of diminished supplying power per unit mass if our hypothesis is correct, will cause a reduction in the supply of nutrients to the shoots, and hence in the synthesis of materials later to be used in the growth of both shoots and roots.

TABLE 2.

(Duration of experiment $5\frac{1}{2}$ days.)

Treatment.	Resp.*	Final D.W. (gm.).		Ion Uptake in gm.-ions $\times 10^{-5}$/gm. D.W. of Rts.				
		Rts.	Shts.	$K^{\cdot}$	$Ca^{\cdot\cdot}$	SO_4''	NO_3'	Cl'
21% O_2	0·846±·366	0·1504	0·5568	215	56·8	47·8	841	58·0
"	1·015±·320	0·1720	0·8310	223	92·6	47·8	1112	61·9
"	0·814±·284	0·1160	0·5604	224	109·0	65·4	878	82·4
"	0·830±·529	0·1644	0·6962	137	40·0	48·6	842	73·2
"	1·073±·838	0·1326	0·6036	309	11·2	38·8	968	43·9
Mean	**0·916**	**0·147**	**0·650**	**222**	**61·9**	**49·7**	**928**	**63·7**
S.D.	—	±0·023	±0·116	±61	±39·5	±9·7	±115	±14·7
2–3% O_2	0·340±·185	0·1534	0·7314	128	20·8	38·6	459	59·7
"	0·433±·539	0·1566	0·7933	31	5·2	32·9	382	45·9
"	0·460±·220	0·0949	0·5664	70	98·3	41·5	378	3·2
"	0·382±·142	0·1491	0·6484	129	5·5	11·2	589	80·5
"	0·567±·515	0·1442	0·6535	260	66·0	46·4	454	27·5
Mean	**0·436**	**0·140**	**0·679**	**124**	**39·2**	**34·1**	**452**	**43·4**
S.D.	—	±0·025	±0·087	±87	±41·3	±11·7	±85	±29·6
P	—	—	—	7%	40%	2%	<1%	20%

Initial dry weight of roots = 0·161 ± 0·030 gm.
" " " shoots = 0·352 ± 0·055 gm.

* In mgm. CO_2/gm.-hr. Mean and standard deviation of three observations of approximately four hours each.

A comparison of supplying activity in the two treatments in terms of intake per gramme of absorbing root-system might enable us to determine whether the data are reconcilable with our hypothesis. Comparison for such purpose would be invalid if there were any differential effect on rate of intake caused by the shoot. On the basis of the hypothesis this would not be expected

for, since the shoots were exposed to a uniform oxygen concentration, it is to be presumed that they did not differ significantly among themselves in rate of respiration;[9] one would therefore expect them to tend to have the same ionic concentrations in their cells. It might be assumed on these grounds that the growth rate was limited by the rate of supply of ions in the case of the low respiration series, so that reduced growth rate is a result rather than a cause of reduced rate of ion intake. If the hypothesis suggested is true, we might therefore anticipate in this series a reduced rate of ion intake per unit weight of absorbing region of root system.

As the absorbing region of the root-system could not be determined experimentally, the total dry weight of the root-system was taken as a measure thereof, on the assumption that the variation in form among the roots in the various replicates was negligible. The further assumption was made that the increase in size of the roots was approximately linear during the time of the experiment, and the arithmetic mean of the final and the estimated initial dry weights was taken as a basis for the expression of ion intake in each of the replicates. The small standard deviation of the initial dry weight value was neglected.

TABLE 3.

(Duration of experiment 6½ days.)

Treatment.	Resp.*	Final D.W. gm.).		Ion Uptake in gm.-ions × 10^{-5}/gm. D.W. of Rt.			
		Rts.	Shts.	K^{+}	Ca^{++}	SO_4^{--}	Cl^{-}
21% O_2	3·96	0·1185	0·6440	1192	395	30·4	13·3
,,	3·58	0·1633	0·6599	812	307	27·5	9·5
,,	3·09	0·1981	0·8831	807	237	29·8	8·6
,,	3·27	0·1179	0·6805	1144	456	32·4	15·7
,,	4·09	0·1423	0·7350	862	379	9·0	9·5
,,	5·54	0·1517	0·6179	641	279	13·6	10·9
Mean	**3·92**	**0·149**	**0·703**	**910**	**342**	**23·8**	**11·3**
S.D.	—	±0·030	±0·96	±214	±82	±9·9	±2·7
2–3% O_2	1·90	0·1465	0·7177	571	422	10·5	8·2
,,	1·35	0·1322	0·6284	620	319	4·8	10·6
,,	2·13	0·1206	0·5916	764	131	7·5	6·2
,,	2·76	0·1443	0·5126	420	396	11·6	7·3
,,	1·88	0·1267	0·6314	701	385	8·1	11·8
,,	2·42	0·1495	0·6407	549	267	28·4	10·1
Mean	**2·07**	**0·137**	**0·620**	**604**	**320**	**11·8**	**9·0**
S.D.	—	±0·012	±0·067	±121	±109	±8·5	±2·1
P	—	—	—	1%	70%	5%	13%

Initial dry weight of roots = 0·177 ± 0·025
,, ,, ,, shoots = 0·579 ± 0·068

* Results of a single set of observations of four hours' duration.

[9] Löweneck (1930) has demonstrated the independence of the metabolic activities in stem and root.

An alternative assumption would have been that the relative growth rate was constant during the experimental time, in which case the geometric rather than the arithmetic mean would have been taken. Actually our knowledge of the growth of cereal plants at the age of five weeks suggests that the relative growth rate was already falling, not so rapidly that the growth-time curve was linear, but so that it had a positive curvature less steep than that of an exponential curve. The true mean may therefore be expected to lie between the arithmetic and geometric means.

Examination of the data expressed in this way in Table 1 shows that the mean intake of various ions in the high respiration series is in general greater than that in the low respiration series. To test the significance of the difference between the means we have applied Fisher's t test. In the tables are given the resulting values of P, the probability that such a difference in means could be obtained by chance. These values, taken as a whole, suggest that decreasing respiration is associated with a significant decrease in ion intake per unit mean weight of root-system.[10]

This conclusion depends on the assumption we have made that there is no differential effect on intake produced by the sub-aerial portion of the plant. But the reduced growth of the tops in the low respiration series may be due to some other factor than reduced supply of ions, for example, to the effect of reduced root respiration on some correlation mechanism existing between shoot and root. In this case it might be concluded that reduced ion intake was due to diminished growth of the plant. To eliminate this possibility experiments were carried out over shorter time intervals, in order to reduce the difference in the final size of the two series. Results of two such experiments are given in Tables 2 and 3.

Table 3 shows much less difference in maximum size attained, and Table 2 no significant difference at all. In both cases there has been a slight reduction in dry weight of roots, barely significant in Table 2. This may be attributed to the ever-present tendency of the plants to produce adventitious roots to take the place of the primary system. These adventitious roots were removed as they appeared; their formation would, however, tend to deflect the flow of synthesized materials from the leaves to the primary root-system, causing this to suffer reduction in carbohydrate content through catabolic breakdown. Since growth appeared not to take place in the root systems, ion intake has been expressed in terms of their final weight.

Table 3 adds some confirmation to the results obtained in Table 1. Table 2 gives the ion intake for plants whose growth has not been significantly influenced by the treatment; and here again the tendency is evident for ion intake to be less with low root respiration.

[10] It must also be realized that in taking the arithmetic mean of the initial and final root weights, we have obtained a minimal measure of the difference in uptake between the two series. Could we have obtained the true mean, which is nearer the geometric mean, the differences in uptake would have been greater and the values of P smaller.

The variability among the replicates within treatments is undoubtedly high in all experiments. This is probably due to variability in material, and in particular to the fact that total dry weight of the root system is not a true measure of the absorbing region thereof. The three sets of data given, however, added to similar unpublished sets, leave no doubt as to the validity of the general conclusions drawn from them, despite the high value of P in individual cases of some ions.

It is not suggested that the hypothesis developed in the early portion of the paper is the true interpretation of these data; doubtless they could be interpreted on other lines; they are presented as of interest, however, because they agree with a hypothesis that was developed from other considerations.

SUMMARY.

A hypothesis is developed wherein four mechanisms are suggested as causing intake of ions by the plant from its environment. It is shown that in terms of this hypothesis the rate of intake may be dependent on the rate of respiration of the absorbing region of the root. Some data are then presented which agree with the view that such a relationship holds.

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ALTERATION OF SEX RATIO IN THE "FLOUR BEETLE" *TRIBOLIUM CONFUSUM* DUVAL, FOLLOWING STARVATION OF NEWLY HATCHED LARVAE

by

F. G. HOLDAWAY AND H. FAIRFIELD SMITH

(From the Divisions of Economic Entomology and Plant Industry, C.S.I.R., Canberra[1]).

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INTRODUCTION.

In the course of studies on the effect of atmospheric moisture on the growth of populations of the "flour beetle," *Tribolium confusum* (Holdaway (1932)), it was observed that newly-hatched larvae kept for a period without food had a sex ratio different from that of larvae given food on hatching. Experiments were therefore planned to determine whether the change in sex ratio was proportional to the period of starvation.

The results indicate that the changes in sex ratio observed cannot be explained on a basis of differential mortality, that they are not progressive and proportional to the period of starvation, and that with starvation for 2 or 3 days there is a preponderance of females. The last result is of interest, since the trend of most previous work has been to indicate that adverse nutrition favours the production of males.

EXPERIMENTAL WORK.

Preliminary experiments indicated that larvae could be starved for more than three days without fear of high mortality.[2] The plan of experiments on starvation was as follows: Eggs laid during a twenty-four hour period in a

[1] The work here reported was carried out by one of us (F.G.H.) in the Division of Entomology, University of Minnesota, in 1928, during the tenure of a research studentship in entomology, Commonwealth Council for Scientific and Industrial Research. Facilities for the work were provided by Dr. R. N. Chapman, formerly of the University of Minnesota, now Dean of the Graduate School of Tropical Agriculture, University of Hawaii. The information was originally incorporated in the manuscript "Environment and Sex" referred to elsewhere (Joyet-Lavergne, 1931). The mathematical analysis has been made by the other of us (H.F.S.).

[2] General remarks on the technique for handling *Tribolium* are given elsewhere, Chapman (1918, 1928), Holdaway (1932).

culture at constant temperature and humidity were removed from the flour by sieving. They were divided into four equal lots, and each lot placed without flour in vessels open to the air. The containers were labelled control, 1 day starvation, 2 days' starvation, and 3 days' starvation respectively, and, with the eggs contained therein, were placed again under the same conditions as those at which the eggs had been laid. Hatching occurs over a period of two days. The eggs were therefore examined every twelve hours, and the newly hatched larvae removed and submitted to the starvation decided on for the batch from which they issued; in each case starvation took place in vessels exposed to moist air. The actual starvation in the respective batches was thus 0–$\frac{1}{2}$ day (control), $\frac{1}{2}$–$1\frac{1}{2}$ days, $1\frac{1}{2}$–$2\frac{1}{2}$ days, and $2\frac{1}{2}$–$3\frac{1}{2}$ days.

The same stock of whole wheat flour was used throughout the experiments, and during each experiment all stages of the insect were exposed to constant temperature, humidity, and light. The sole difference between the treatment given to each of the four batches was in the duration of the starvation period. The results obtained from the complete experiments, together with those obtained from less complete experiments, are given in Table 1 and represented graphically in fig. 1. Sex can be recorded at pupation by means of the genitalia.

TABLE 1.

Sex Ratios Obtained when Newly-hatched Larvae were Submitted to Different Periods of Starvation.

(All experiments at 27° C.)

Experiment.	Period of starvation (days).	Relative humidity.	Number of larvae.	Number pupated.	Male.	Female.	Sex ratio (males as % of total).	Larval Mortality.	% Mortality.
1	0 (Control)	50%	150	148	77	71	52·0	2	1·4
	1	50%	150	150	88	62	58·6	0	0·0
	2	50%	150	147	66	81	44·9	3	2·0
	3	50%	150	148	68	80	45·9	2	1·4
2	0 (Control)	75%	181	177	93	84	52·5	4	2·2
	1	75%	183	177	100	77	56·5	6	3·3
	2	75%	188	177	84	93	47·5	11	5·9
	3	75%	174	162	81	81	50·0	12	6·9
3	0 (Control)	Same as in experiment 1.							
	2	50%	150	148	69	79	46·7	2	1·4
	3	50%	150	147	78	69	53·1	3	2·0
4	0 (Control)	75%	316	310	158	152	51·0	6	1·9
	3	75%	160	155	64	91	41·3	5	3·1
5	0 (Control)	75%	116	109	56	53	51·4	7	6·0
	1	75%	108	106	58	48	54·7	2	1·9
		(Starvation at 1± %).							

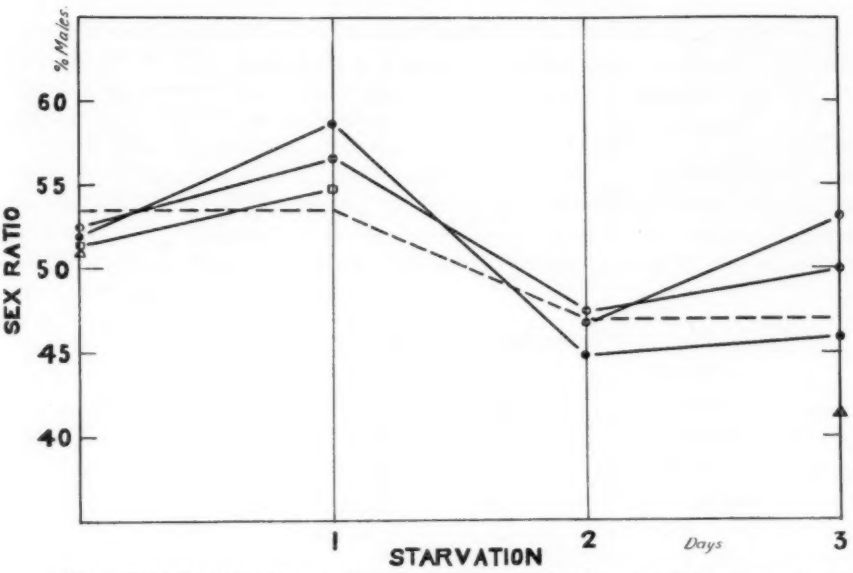

Fig. 1. Showing the change in sex ratio following starvation of newly hatched larvae of *Tribolium confusum* Duval. Different experiments are indicated by different systems of marking. Different readings in the same experiment are joined by solid lines. The dotted line represents the hypothesis which most satisfactorily explains the observations.

ANALYSIS OF THE DATA.

To determine whether the humidity at which the starvation occurred had an effect on the resultant sex ratios, separate contingency tables have been constructed for each degree of starvation. (Tables 2a, b, c, d.) By keeping duplicate samples separate an examination of their homogeneity has been made at the same time as the examination for humidity effect.

TABLES 2a, b, c, d.

To Determine whether the Humidities at which Starvation Occurred had an Effect on the Resultant Sex Ratios.

TABLE 2a.

All Control Samples (0 Day Starvation).

Humidity		50%	75%	75%	75%	Total.
Observed	♂♂	77	56	93	158	384
	♀♀	71	53	84	152	360
Total		148	109	177	310	744
Calculated	♂♂	76·4	56·2	91·4	160·0	384
	♀♀	71·6	52·8	85·6	150·0	360
0—C		±0·6	∓0·2	±1·6	∓2·0	
		χ^2=·121	n=3	P=·75		

TABLE 2b.

All Samples for 1 Day of Starvation.

Humidity		1%	50%	75%	Total.
Observed	♂♂	58	88	100	246
	♀♀	48	62	77	187
Totals		106	150	177	433
Calculated	♂♂	60·2	85·2	100·6	246
	♀♀	45·8	64·8	76·4	187
O—C		∓2·2	±2·8	∓0·6	

$\chi^2 = \cdot 407$ n=2 P=·81

TABLE 2c.

All Samples for 2 Days of Starvation.

Humidity		50%	50%	75%	Total.
Observed	♂♂	66	69	84	219
	♀♀	81	79	93	253
Totals		147	148	177	472
Calculated	♀♀	68·2	68·7	82·1	219
	♂♂	78·8	79·3	94·9	253
O—C		∓2·2	±0·3	±1·9	

$\chi^2 = \cdot 2168$ n=2 P=·89

TABLE 2d.

All Samples for 3 Days of Starvation.

		50%	50%	75%	75%	Total.
Observed	♂♂	68	78	81	64	291
	♀♀	80	69	81	91	321
Totals		148	147	162	155	612
Calculated	♂♂	70·4	69·9	77·0	73·7	291
	♀♀	77·6	77·1	85·0	81·3	321
O—C		∓2·4	±8·1	±4·0	∓9·7	

$\chi^2 = 4 \cdot 776$ n=3 P=·19

In all four tables the deviations are shown to be such as would be expected to arise due to chance alone. It therefore appears justifiable to consider that all samples for each period of starvation are homogeneous, and to use the totals for each period in examining the effect of starvation.

TABLE 3.

Numbers of each Sex found after each Period of Starvation

		Period of Starvation. 0	1 day.	2 days.	3 days.	Total.
Observed	♂♂	384	246	219	291	1140
	♀♀	360	187	253	321	1121
Totals		744	433	472	612	2261
Calculated	♂♂	375·1	218·3	238·0	308·6	1140
	♀♀	368·9	214·7	234·0	303·4	1121
O—C		±8·9	±27·7	∓19·0	∓17·6	

$\chi^2 = 12 \cdot 57$ n=3 P<·01

The first question to be examined is whether the observed variation in the sex ratio with different degrees of starvation is statistically significant. To test this a contingency table (Table 3) has been constructed. This gives $P < 0{\cdot}01$, so the variation must be regarded as significant.

The next question is to determine the nature of the variation in so far as this can be determined by the data available. The first hypothesis which occurs to one is that there is a steady change in sex ratio for successive degrees of starvation. The appearance of the data does not support this, but to complete the analysis a straight regression line for sex ratio on period of starvation has been calculated.

This line is found to be

$$y = 0{\cdot}531 - 0{\cdot}0193\,t$$

where "y" is the proportion of males and "t" is the period of starvation in days. On applying the test of Goodness of Fit between observations and values calculated from this regression it is found that $\chi^2 = 7{\cdot}53$, $n = 2$, $P = 0{\cdot}025$. It therefore seems unlikely that such an hypothesis adequately describes the observations.

The major change appears to take place between one-day and two-day periods of starvation. We have therefore taken as a second hypothesis that all of the change in sex takes place at that time. To test this hypothesis the data have been grouped into two contingency tables, one for zero and one day of starvation, and the other for longer periods. Table 4 is the result, and it indicates that if this hypothesis be true, deviations greater than have occurred could be expected in about one out of five such experiments. It is therefore considered that within the limits of experimental error this hypothesis can explain the observations. This hypothesis is represented by the dotted line in fig. 1.

TABLE 4.

To test the hypothesis that the observed Sex Ratios can be explained by a change occurring only between the one- and two-day periods of starvation.

		Period of starvation.					
		0	1 day.	Total of 0 and 1 day.	2 days.	3 days.	Total of 2 and 3 days.
Observed	♂♂	384	246	630	219	291	510
	♀♀	360	187	547	253	321	574
Totals		744	433	1,177	472	612	1,084
Calculated	♂♂	398·2	231·8		222·1	287·9	
	♀♀	345·8	201·2		249·9	324·1	
O—C		±14·2			±3·1		

$\chi^2 = 3{\cdot}116$ n=2 P=·21

This does not of course mean that it has been proved that no other change takes place. Within the limits of experimental error this hypothesis is in agreement with the observations, but other changes may take place which the present data are inadequate to detect.

The largest contribution to χ^2 in Table 4 is made by the observations for one day of starvation. A comparison between this class and the control (first half of Table 4) indicates that a greater difference in sex ratio than that observed could be expected by chance, on the average, once in eleven experiments of this size ($P = 0{\cdot}09$). This deviation cannot be considered statistically significant, but for a reason to be mentioned it might be regarded as indicative of a tendency for a higher proportion of males to be produced after starvation for one day.

Table 5 shows the proportions of males obtained from all the data for each degree of starvation. The difference between the sex ratio for 2 and 3 days (0·470) and that for the control (0·516) is $0{\cdot}046 \pm 0{\cdot}0238$; $P = 0{\cdot}05$.

TABLE 5.

Proportions of Males Observed.

Period of starvation.	0	1 day.	2 days.	3 days.
Proportion of males	·516	·568	·464	·475
Mean proportion for successive periods	·535		·470	

To complete the analysis it is necessary to consider whether the results can be explained on the basis of differential mortality. The total mortality was:

Period of starvation.	0	1 day.	2 days.	3 days.
No. of larvae died	19	8	16	22
Per cent. of larvae died	2·49	1·81	3·28	3·47

If it be assumed that all that died were either males or females we get sex ratios as follows:

Period of starvation.	0	1 day.	2 days.	3 days.
Assuming only ♀♀ die	·503	·558	·449	·459
Assuming only ♂♂ die	·528	·576	·482	·494

These are no more uniform than the observed ratios.

To construct an hypothesis involving differential mortality at different degrees of starvation we first note that since the mortality after one day of starvation is lower than that for no starvation, the differences can only be put down to chance. It is therefore necessary to assume that for these two periods the mortality is equal. We shall assume it to be the weighted mean, viz., 2·24%, and that this is due entirely to the death of females. For two and three days of starvation the mortality and sex ratio are practically equal, and so we shall deal only with the means. The mean mortality is 3·39%. By the preceding assumption 2·24% have been assumed to be female, the remaining 1·15% we shall assume to be male. This hypothesis yields:

Period of starvation	0	1 day.	2 and 3 days.
♂♂	384	246	523
♀♀	379	195	599
Proportion of ♂♂	·503	·558	·466
Proportion (mean of 0 and 1 day)	·523		

This is little better than the original observations. The difference between the proportion of males for 0 and 1 day and for 2 and 3 days is $0{\cdot}058 \pm 0{\cdot}0207$; $P = 0{\cdot}005$. It is therefore concluded that the differences in sex ratio noted for different periods of starvation cannot be explained by differential mortality.

DISCUSSION.

The results given above indicate that the sex of *Tribolium* can be changed by starvation of first instar larvae. The results are definite for a change from male to female. Most of the work published on a possible relation between nutrition and sex in animals has suggested that with adverse nutrition there is an excess of males. Evidence, including some from the Insecta, quoted by Geddes and Thomson (1901) is of this kind.

Malaquin (1901) with parasitic Copepods, Christie (1929) with *Mermis subnigrescens*, and Holdaway and Smith (1932) with *Alysia manducator* have obtained results which suggest that adverse nutrition tends to the production of males. The results of Chewyreuv (1913), who obtained male Ichneumonids from small and female from large host larvae, suggest a similar conclusion, although Chewyreuv interpreted his results otherwise. Wheeler (1910) found that when the number of *Xenos* parasites in *Polistes* was more than four they were always males. Jarvis (1914) found a high proportion of male blowflies emerging from small puparia. Herms (1928) with *Lucilia sericata*, and Weidling (1918) with *Calliphora erythrocephala*, obtained similar results, but concluded that the figures obtained were probably due to differential mortality. It should be mentioned in passing that M. J. Mackerras, at Canberra, found no significant difference in the sex ratio of *Lucilia cuprina* and *L. sericata* with starvation. The results recorded for *Tribolium* are thus contrary to the generalization that with adverse nutrition there is a tendency towards excess of males. There are some results in which the direction of change is comparable to that in *Tribolium*. For example, Geoffrey Smith (1909) found that male crabs parasitized by *Sacculina* tended to assume female characters. *Andrena* males parasitized by Strepsiptera were found by Pérez (1886) to take on certain female characteristics, and Kornhauser (1919) found that male Membracids parasitized by Strepsiptera also assumed female characteristics. Champy (1921) found that poverty of food led to the appearance of female oocytes in a male *Triton*. Further, there is some evidence which shows that in the same host species a stylopized male may show female characteristics, while a stylopized female may show male characteristics (*vide* Salt (1927)). The effects of parasitism on the sexual characteristics of the host are possibly explainable in part or in whole on a nutritional basis. Nevertheless, the diversity of these results suggest the desirability of considering the relation of nutrition to sex from the point of view of the biochemical changes in the body fluids associated with adverse nutrition, rather than merely from the point of view of favourable or adverse nutrition.

In all the experiments on *Tribolium* there were more males after starvation for one day than there were in the control. It was therefore formerly concluded (Holdaway (1930)) that the results indicated a change first to excess of males with one day starvation, followed by a change to excess of females with two and three days' starvation. However, as has been shown above in the analysis, the figures are not adequate for concluding with reasonable certainty that there is an increase in the proportion of males after one day of starvation. But since other evidence suggests that adverse nutrition may lead to the production of males, we cannot on the basis of the present analysis rule out the possibility that slight starvation produces males, while severe starvation produces females. If the increase in the proportion of males following one day's starvation could be established statistically, then the change from excess of males to excess of females which occurs between one day and two days' starvation would assume even greater significance than the change indicated when the control and one day starvation are taken together, as in Table 4.

ACKNOWLEDGMENTS.

Our thanks are due to Mr. F. Yates, of Rothamsted, who suggested the method used in testing differences which might be due to changing humidities before going on to test for the effect of starvation on the combined data. We are also indebted to Miss F. E. Allan, of Canberra, for her ever-ready willingness to enter into helpful discussion on statistical matters.

SUMMARY.

The results of experimental work on starvation of newly-hatched larvae of the "flour beetle," *Tribolium confusum* Duval, are given, together with a mathematical analysis of the data. With starvation for two days or for three days there is an excess of females. The results cannot be explained on a basis of differential mortality of the sexes, and the change has been shown to be statistically significant.

In all the experiments a higher proportion of males was recorded with starvation for one day. These latter figures, however, are not statistically significant, and hence do not permit of a definite conclusion. But since evidence published for other organisms indicates that adverse nutrition favours the production of males there is a possibility that this may also be the case in *Tribolium* with starvation for one day, although a more prolonged starvation for two or three days results in excess of females.

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NOTE ON THE OCCURRENCE OF A WHITE-EYED MUTANT RACE OF *LUCILIA CUPRINA* WIED

by

M. J. MACKERRAS

(Division of Economic Entomology, Commonwealth Council for Scientific and Industrial Research, Canberra, F.C.T.)

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The stock in which this mutation appeared, was derived originally from a few maggots obtained from a struck sheep in Canberra in October, 1931. As the number of maggots was small, they were probably the result of a single oviposition. The flies were allowed to breed, and during the summer and autumn 1931-1932 a large stock was maintained in the insectary for work on experimental blowfly strike in sheep. In April, 1932, a small number of flies was transferred to the warm temperature room and successive generations maintained there in cages throughout the winter at a temperature varying from 20°C. to 22°C. This room (a cellar) is warmed by an electric radiator controlled by a thermo-regulator and mounted in front of a fan, and is lighted by day by a 1,000 watt lamp. No sunlight can enter this room. The flies were fed on cane sugar, dates, and water. Fresh liver was supplied every few days. Under these conditions this species lives well and breeds prolifically.

The mutation was first noticed in June, 1932, in a batch of flies which had been subject to extreme starvation in the larval period. Out of 288 very small flies 13, or 4·5%, were white-eyed. These flies were very stunted and feeble, and, although the white-eyed individuals were segregated from the remainder, and much care given to them, they died without producing any fertile eggs. About two months later the mutation appeared again among the progeny of the original stock. Unfortunately no record of the number of generations intervening had been kept, but it was probably the second generation after the first appearance of the mutation. This time the white-eyed flies were of normal size, and, when segregated, bred freely together, producing only white-eyed progeny. The character has bred true through three generations. These white-eyed flies show another peculiarity, in that the contents of their Malpighian tubes are colourless, whereas in normal red-eyed flies the tubes appear bright yellow. Lack of eye-pigment has also been found associated with lack of pigment in the contents of the Malpighian tubes in *Psychoda alternata* (Turner (1923)).

The Result of Crossing Mutants with Normal Flies. When a red-eyed fly is mated with a white-eyed fly, the progeny is all red-eyed. This is true whichever way the cross is made. On breeding these flies together, red-eyed and white-eyed flies appear in the next generation approximately in the ratio of 3 : 1, which is what one would expect if dealing with a simple Mendelian factor.

In one experiment a normal red-eyed male was paired with a newly-emerged white-eyed female. Only one batch of eggs was laid by this fly. The resulting flies (F_1 generation) were all red-eyed. These were allowed to breed together and the first few thousand eggs laid were removed and bred through. A count of the F_2 generation gave the following figures:

Red-eyed females ..	1,775	White-eyed females ..	615
Red-eyed males ..	1,717	White-eyed males ..	597
Total red-eyed flies ..	3,492	Total white-eyed flies	1,212

$$\frac{\text{Red-eyed flies}}{\text{White-eyed flies}} = \frac{3,492}{1,212} = \frac{2 \cdot 88}{1 \cdot 0}$$

The difference between this ratio 2·88 : 1 and the Mendelian ratio of 3 : 1 is statistically within the limits of variation due to chance.

In another experiment a newly-emerged red-eyed female was paired with a white-eyed male. Five batches of eggs were produced and all gave rise to red-eyed flies. One batch of these flies (F_1 generation) was bred together and eggs removed and bred through under similar conditions to the previous experiment. A count of the resulting F_2 generation gave the following figures:

Red-eyed females ..	1,409	White-eyed females ..	409
Red-eyed males ..	1,483	White-eyed males ..	403
Total red-eyed flies ..	2,892	Total white-eyed flies	812

$$\frac{\text{Red-eyed flies}}{\text{White-eyed flies}} = \frac{2,892}{812} = \frac{3 \cdot 56}{1 \cdot 0}$$

This ratio is statistically outside the limits of chance variation from 3 : 1. This may be explained by a differential mortality in the larval stage, supposing the white-eyed strain to be more susceptible to an adverse environment. It was noted that there were more undersized flies in this batch than in the previous one, showing that competition had been keener.

The red-eyed character is thus dominant and the white-eyed recessive. If, following Morgan, we let (w) stand for the recessive gene for white-eyes and (W) for its allelomorph, the normal dominant gene for red eyes, we may represent a normal red-eyed fly as (WW), a white-eyed fly as (ww), and a hybrid red-eyed fly as (Ww). The crosses may then be written as follows:

P_1 .. (WW) x (ww)
↓
F_1 .. (Ww)
↓
F_2 .. (WW), (Ww), (Ww), (ww)

i.e., three red-eyed flies to one white-eyed fly.

The Result of Crossing a Hybrid and a Recessive. When a hybrid (red-eyed) female was mated with a white-eyed male, the progeny resulting from two batches of eggs consisted of:

Red-eyed females ..	98	White-eyed females ..	108
Red-eyed males ..	116	White-eyed males ..	103
Total red-eyed flies ..	214	Total white-eyed flies	211

$$\frac{\text{Red-eyed flies}}{\text{White-eyed flies}} = \frac{214}{211} = \frac{1{\cdot}01}{1{\cdot}0}$$

This is very close to the theoretical ratio of 1 : 1. The cross in this case is:

P_1 .. (Ww) x (ww)
↓
F_1 .. (Ww), (ww)

i.e., one red-eyed fly to one white-eyed fly.

The gene for eye-colour is evidently not sex-linked in *Lucilia cuprina*, as it is in *Drosophila melanogaster*. In the latter insect the results of crossing a white-eyed fly with a red-eyed one differ according to the sex of the white-eyed fly. It has been shown by Morgan (1919) that these variations may be explained by the hypothesis that the gene for eye-colour is linked with the X (sex) chromosome.

The white-eye character in *Lucilia cuprina*, then, behaves as an ordinary Mendelian recessive. It is correlated with an absence of pigment in the contents of the Malpighian tubes. These characters are the outward manifestation of some deep-seated departure from the normal metabolism of the insect. The experiments reported here are merely of a preliminary nature, and there is a wide field for work on the genetics and physiology of the insect.

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THE EFFECTS OF MODERATE OVERDOSAGE OF VITAMIN D AND OF VITAMINS A+D ON THE GROWTH RATE AND LONGEVITY OF THE WHITE MOUSE

PART II

by

THORBURN BRAILSFORD ROBERTSON, HEDLEY R. MARSTON, MARY C. DAWBARN, J. W. WALTERS, AND J. D. O. WILSON

(From the Commonwealth Council for Scientific and Industrial Research, Division of Animal Nutrition, University of Adelaide, South Australia).

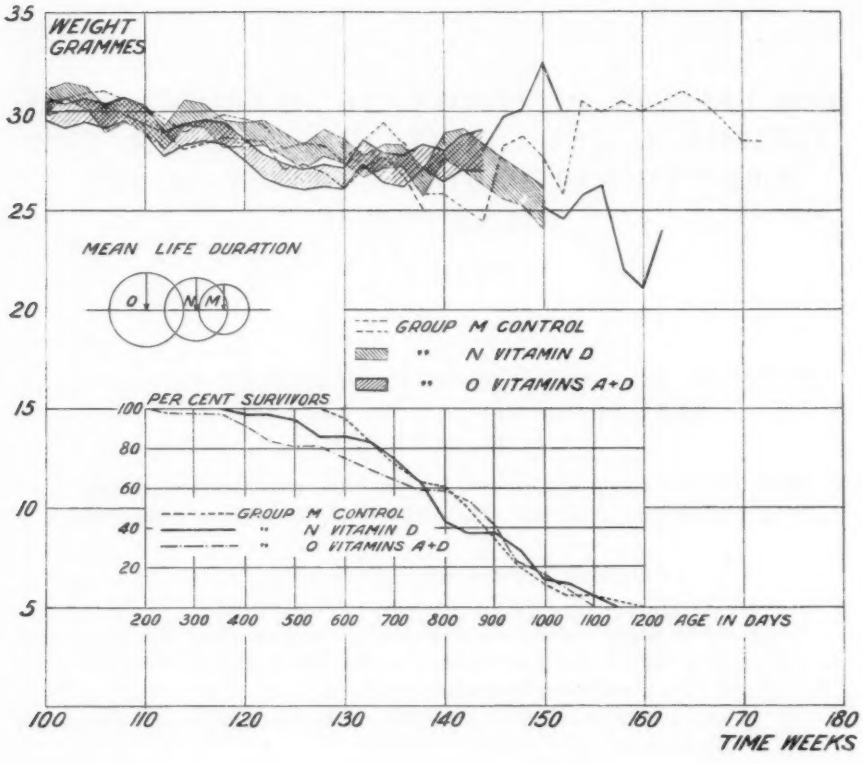
(Submitted for publication 4th January, 1933.)

In a previous paper (Robertson, *et al.*, 1932) the conduct of this experiment was described in some detail, and the results obtained during the first 100 weeks of the lives of the animals were discussed.

The growth curves from 100 weeks of age to the death of the last animal are given here, together with the mortality statistics.

A few words must suffice to recapitulate the data of the earlier paper. Three groups, each of 36 male animals, were fed on a mixed diet. The individuals of one group (N) received daily a supplement of 50 rat-units of vitamin D dissolved in 0·05 c.c. of olive oil, while those of the second group (O) received 50 rat-units of vitamin D and 460 rat-units of vitamin A dissolved in the same quantity of oil. The individuals of the third group (M), which acted as a normal control, received merely the daily dose of 0·05 c.c. of the solvent, olive oil.

The growth of the mice in group N was favourably influenced over the first 30 weeks of the experiment. The beneficial effect of excess of vitamin D was entirely counteracted by administering simultaneously an excess of vitamin A. After 35 weeks of age the mean weight of the individuals comprising the three groups was not significantly different. Group N, however, maintained an apparent though not statistically significant superiority in weight over the others until they were about 90 weeks of age.



From this time until death the weight of the animals was not affected by the difference in treatment.

If we consider the mortality statistics it becomes apparent from the survival curves that, while early deaths were less frequent in control group M than in either of the groups O or N, after the lapse of 750 days the total number of deaths in each group was approximately equal. A calculation revealed the fact that the mean duration of life of animals still existing at 750 days did not differ significantly in the three groups.

The life duration of mice dying before 750 days of age differed appreciably in groups M and O, with group N occupying an intermediate position.

While the response to moderate overdosage of vitamin D in an earlier experiment was an insignificant increase in longevity (*loc. cit.*, 1932), the life duration of the animals receiving similar treatment in the present experiment was slightly

less than that of the controls, and one may conclude that an excess of vitamin D of this order, administered throughout life, has no appreciable effect on longevity. When, however, a large excess of vitamin A was simultaneously administered with the overdosage of D the expectation of life of the young animals was reduced. The expectation of life of those animals which survived to 750 days was not significantly altered.

TABLE 1.

Group M. Controls.

Number of Animals.	Age in weeks.	Mean weight in Grammes.	Probable error in Grammes.
25	100	30·14	·35
24	102	30·38	·31
23	104	30·50	·46
22	106	30·66	·42
22	108	30·23	·39
22	110	29·86	·42
22	112	29·30	·36
22	114	28·57	·42
19	116	28·97	·53
18	118	29·28	·56
17	120	29·12	·52
17	122	28·94	·42
15	124	27·63	·57
13	126	27·88	·55
11	128	27·64	·68
11	130	26·86	·73
9	132	27·89	·70
8	134	28·62	·83
7	136	27·64	·84
6	138	25·83	·80
4	140	25·87	—
3	142	25·17	—
3	144	24·50	—
2	146	28·25	—
2	148	28·75	—
2	150	27·55	—
2	152	25·75	—
1	154	30·50	—
1	156	30·00	—
1	158	30·50	—
1	160	30·00	—
1	162	30·50	—
1	164	31·00	—
1	166	30·00	—
1	168	29·50	—
1	170	28·50	—
1	172	28·50	—

TABLE 2.

Group N. Vitamin D.

Number of Animals.	Age in weeks.	Mean weight in Grammes.	Probable error in Grammes.
26	100	30·50	·54
24	102	31·02	·47
24	104	30·71	·55
23	106	29·61	·64
21	108	30·07	·56
21	110	29·55	·66
18	112	28·69	·62
15	114	29·97	·65
15	116	29·87	·55
14	118	28·93	·70
14	120	28·89	·63
13	122	28·92	·59
13	124	28·38	·55
13	126	27·77	·59
13	128	28·46	·67
13	130	28·08	·62
13	132	27·23	·56
10	134	27·70	·59
9	136	27·72	·52
8	138	26·87	·66
6	140	28·25	·66
5	142	28·10	1·08
5	144	27·30	1·01
4	146	26·62	—
4	148	26·12	—
4	150	25·12	—
2	152	24·50	—
2	154	27·75	—
2	156	26·25	—
2	158	22·00	—
2	160	21·00	—
1	162	24·00	—

TABLE 3.

Group O. Vitamins A+D.

Number of Animals.	Age in weeks.	Mean weight in Grammes.	Probable error in Grammes.
22	100	30·07	·53
21	102	29·74	·61
21	104	30·19	·57
21	106	29·79	·59
21	108	30·14	·61
21	110	29·52	·63
21	112	28·36	·61
20	114	28·85	·55
20	116	29·05	·56
20	118	28·87	·53
20	120	28·10	·54
19	122	27·26	·60
19	124	26·82	·52
16	126	26·59	·54
15	128	26·77	·53
14	130	26·64	·49
10	132	27·90	·61
8	134	27·12	·77
8	136	27·00	·80
7	138	27·71	·65
6	140	27·25	·86
6	142	27·91	·88
6	144	27·99	1·09
4	146	29·75	—
4	148	30·12	—
2	150	32·50	—
1	152	30·00	—

MORTALITY STATISTICS.

(Accidental deaths excluded.)

At Age in Days.	Percentage of Survivors. Group M. Control.	Group N. Vitamin D.	Group O. Vitamins A+D.
200	100	100	100
250	100	100	97·2
300	100	100	97·2
350	100	100	97·2
400	100	97·1	91·7
450	100	97·1	83·3
500	100	94·3	80·6
550	100	85·7	80·6
600	94·3	85·7	75·0
650	82·9	82·9	69·4
700	71·4	74·3	63·9
750	62·9	62·9	58·3
800	60·0	42·9	58·3
850	48·6	37·1	52·8
900	34·3	37·1	41·7
950	20·0	28·6	22·2
1,000	11·4	14·3	16·7
1,050	5·7	11·4	8·3
1,100	5·7	5·7	0·0
1,150	2·9	0·0	
1,200	0·0		

Mean Duration of Life.

Group M 825±17 days.
Group N 806±22 „
Group O 771±26 „

Difference between M and O 54±31 days.

Mean Duration of Life of Mice still alive at 750 days.

Group M 924±14 days.
Group N 920±17 „
Group O 944±11 „

Difference between M and O 20±18 days.

Mean Duration of Life of Mice dying before 750 days.

Group M 658± 9 days.
Group N 612±21 „
Group O 529±24 „

Difference between M and N 46±23 days.
N and O 83±32 „
M and O 129±25 „

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THE CHEMICAL COMPOSITION OF THE URINE OF COWS[1]

by

A. T. DANN

(From the Laboratory of Pathology and Bacteriology, Adelaide Hospital, South Australia).

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As little or no published work is to be found on the chemical composition of the urine of cows under Australian conditions, the following observations will help to establish some normal values.

While many determinations of the various constituents have been made separately, only a comparatively few individual samples of urine have been analysed for a number of constituents. Therefore the various determinations will first be considered separately, and then the results of the more complete analyses made on a few urine samples will be given.

Collection and Preservation of Urine Samples. The urines examined were from dairy cows of various breeds, in milk, and at pasture, with usually some hand-feeding. Though 24-hour specimens of urine would have been desirable, their collection was not feasible under field conditions, and all the samples examined were from a single natural urination. Many of the urine samples were examined within a few hours of collection. When examination could not be completed on the same day, toluene was used, or where it would be unobjectionable, formalin.

METHODS AND RESULTS OF ANALYSIS.

a. Animals in the Adelaide District.

The methods used had to be such as could be carried out with a minimum of apparatus, as many determinations were to be made on the field, where few laboratory conveniences were available. In some cases this may have led to slightly lessened accuracy.

Density (at 20°C.). Determined by weighing, or on the field, by the urinometer.

Mean of 27 samples, 1·033: Variation, 1·015 to 1·047.

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[1] The work detailed in this paper was carried out as part of an investigation conducted under the auspices of the Council for Scientific and Industrial Research of the Commonwealth of Australia into the cause of Enzootic Haematuria of cattle.

While the extreme variation is large, only two of the determinations fell below 1·027 and only one above 1·041. Of the low values, one was from a cow known to have calved recently. A similar dilution of the urine accompanying calving has been noted by Keitt (1916).

Reaction. Determined with the Hellige pH colorimeter. The urines were examined within three hours of collection.

Variation of reaction, pH 5·5 to 8·3.

Grazing supplemented with cereal straws gave urines of pH 7·7 to 8·3, while when brewer's grains were given as a supplement, the pH range was from 5·4 to 7·4.

The marked effect of diet on the reaction of cow's urine has been studied by Warth and Ayyar (1930). They found that a diet of cereal straws with concentrates, or of green fodder, gave reactions in the pH range 8·0 to 8·5, while "over-ripe" grasses gave much more acid urines, pH 5·7 to 7·5.

Total Nitrogen. Determined by the micro-method of Koch and McMeekin (1924.)

Mean of 21 samples, 10·1 grms.: Variation, 1·77 to 15·6 grms. per litre. Neglecting a few urines of exceptionally low density, the smallest content of total nitrogen was 7·0 grms. per litre.

Few comparable values are available for dairy cows on balanced ration in other parts of the world, but the above agree well with the results of Krauss (1927), State of New York, U.S.A. (Mean, 9·6 grms.: Variation for 12 determinations, 6·1 to 12·5 grms. per litre.)

Hippuric Acid. Determined by the method of Warth and das Gupta (1928).

This method determines the combined benzoic acid in the urine, and, on the assumption that it is present exclusively in the form of hippuric acid, no account is taken of the probable occurrence of benzoylglycuronic acid.

Mean of 34 samples, 9·46 grms.: Variation, 2·0 to 21·6 grms. "hippuric acid" per litre.

The large variation in the amounts of hippuric acid excreted by ruminants on varying diets has been noted by many workers. Warth and Ayyar (1930) found a variation of 9·6 to 51 grms. per litre, the greatest amounts being in very acid urines accompanying a diet of "over-ripe" grasses. Das Gupta (1932) concludes that, in Indian cattle, benzoic acid is excreted entirely as hippuric.

Urea. Determined by direct nesslerization method of Folin and Youngburg (1929).

Mean of 30 samples, 7·27 grms. urea (3·39 grms. urea nitrogen): Variation, 0·48 to 21·28 grms. urea (0·22 to 9·92 grms. urea nitrogen) per litre.

The lowest values are again associated with urines of very low density. Apart from these, very considerable variations may be found in the urea content

of urines collected on one day from animals in the same herd, and fed on the same diet, e.g., a variation of 6·2 to 21·3 grms., while the variation of density was only 1·0304 to 1·0381.

AMMONIA. Determined by the permutite method of Folin and Bell (1919).

The ammonia content of cow's urine varies very considerably with the reaction of the urine, and so with the diet. Therefore, instead of giving the mean value and variation of ammonia content for all the samples studied, the urines will be grouped according to the diet producing them.

Grazing and Hay. (pH of urine, 7·7 to 8·3.)
Mean of 18 samples, 0·055 grm. ammonia (0·045 grm. ammonia nitrogen): Variation, 0·018 to 0·156 grm. ammonia (0·015 to 0·128 grm. ammonia nitrogen) per litre.

Grazing and Brewer's Grains. (pH of urine, 5·4 to 7·4.)
Mean of 9 samples, 0·516 grm. ammonia (0·424 grm. ammonia nitrogen): Variation, 0·117 to 1·126 grm. ammonia (0·096 to 0·926 grm. ammonia nitrogen) per litre.

Warth and Ayyar (1930) found in the one acid sample which they examined for ammonia a content of 0·94 grm. of ammonia per litre, as compared with 0·03 to 0·07 grm. per litre for the alkaline urines.

CREATININE. Folin's method (1914).

Mean of 21 samples, 1·35 grms.: Variation, 0·36 to 2·41 grms. creatinine per litre.

These values are considerably lower than those of Szendi (1924), who obtained a mean value of 4·41 grms. per litre (variation 2·3 to 8·5 grms. per litre) for 9 urine samples from cows in the neighbourhood of Budapest.

URIC ACID. Method of Benedict and Francke (1922).

Mean of 21 samples, 0·28 grm.: Variation, 0·06 to 0·40 grm. uric acid per litre.

Of these only two urines had a uric acid content of less than 0·20 grm. per litre.

CHLORIDES. (Volhard-Arnold.)

Mean of 14 samples, 9·64 grms. NaCl: Variation, 2·45 to 16·85 grm. per litre.

PHENOLS. Folin and Denis (1915).

Combined Phenols: Mean of 13 samples, 0·60 grm.: Variation, 0·10 to 1·18 grms. per litre.

Free Phenols: Mean of 13 samples, 1·53 grms.: Variation, 0·15 to 2·41 grms. per litre.

Free Phenols., per cent. of total: Mean of 13 samples, 71·6%: Variation, 59% to 87%.

Sulphur. The inorganic and ethereal sulphates and neutral sulphur were determined by Fiske's method (1921). Check determinations made by Folin's (1905-6) gravimetric method gave results in close agreement.

Inorganic Sulphate: Mean of 68 samples, 0·206 grms. S: Variation, 0·006 to 1·161 grms. S per litre.

Ethereal Sulphate: Mean of 68 samples, 0·418 grms. S: Variation, 0·074 to 1·072 grms. per litre.

Inorganic Sulphate: Per cent. of Total Sulphate. Mean of 68 samples, 33%: Variation, 1% to 95%.

Neutral Sulphur: Mean of 7 samples, 0·076 grm. S: Variation, 0·010 to 0·154 grm. S per litre.

It will be noticed that extremely wide variations were found in the quantities of the various groups of sulphur-containing substances. Warth (1930), (1932) has found, as might be expected, that the inorganic sulphate excretion varies considerably with the intake. Of the inorganic sulphate values, 17 were below 0·050 and 8 above 0·400; 3 values of the ethereal sulphate were below 0·100 and 5 above 0·700. For the percentage of total sulphate (inorganic and ethereal) present as inorganic sulphate, 40 were between the limits of 10% and 50%, while 17 values were below 10%, and 11 above 50%.

b. Animals in the Mount Gambier District.

These results are treated separately because they probably illustrate the effect of different climatic and dietetic conditions, Mount Gambier being about 300 miles south-east of Adelaide, the soil being volcanic and the rainfall heavy.[2] In some instances the results depart quite appreciably from those obtained in the Adelaide district.

Density. Mean of 22 samples, 1·028: Variation, 1·014 to 1·043.

Reaction. Variation between pH 6·5 and pH 8·1.

With the exception of two samples of pH 6·5 and 6·6, all urines examined were alkaline, and were, in the main, between pH 7·6 and pH 8·1.

Total Nitrogen. Mean of 36 samples, 5·64 grms.: Variation, 1·60 to 12·27 grms. per litre.

Hippuric Acid. Mean of 36 samples, 12·77 grms.: Variation, 6·6 to 27·6 grms. "hippuric acid" per litre.

Urea. Mean of 24 samples, 4·87 grms. urea (2·28 grms. urea nitrogen): Variation, 1·63 to 8·56 grms. urea (0·76 to 3·99 grms. urea nitrogen) per litre.

[2] This herd was under observation, as some of the animals were suffering from *haematuria vesicalis*. The results recorded were from animals without symptoms of this disease.

AMMONIA. Mean of 22 samples, 0·046 grm. ammonia (0·038 grm. ammonia nitrogen): Variation, 0·011 to 0·109 grm. ammonia (0·009 to 0·090 grm. ammonia nitrogen) per litre.

CREATININE. Mean of 24 samples, 0·59 grm.: Variation, 0·11 to 1·61 grms. creatinine per litre.

URIC ACID. Mean of 23 samples, 0·30 grm.: Variation, 0·11 to 0·55 grm. uric acid per litre.

CHLORIDES. Mean of 12 samples, 10·50 grms.: Variation, 3·62 to 19·37 grms. NaCl per litre.

PHENOLS.

Combined Phenols: Mean of 29 samples, 0·83 grm.: Variation, 0·24 to 2·19 grms. per litre.

Free Phenols: Mean of 29 samples, 1·28 grms.: Variation, 0·46 to 3·08 grms. per litre.

Free Phenols, per cent. of total: Mean of 29 samples, 62·0%: Variation, 53·3% to 76·7%.

SULPHUR.

Inorganic Sulphate: Mean of 65 samples, 0·018 grm.: Variation, 0·003 to 0·067 grm. of S per litre.

Ethereal Sulphate: Mean of 65 samples, 0·264 grm.: Variation, 0·061 to 0·666 grm. of S per litre.

Inorganic Sulphate, per cent. of Total Sulphate: Mean of 65 samples, 6·5%: Variation, 1% to 17%.

Neutral Sulphur: Mean of 10 samples, 0·038 grm.: Variation, 0·010 to 0·067 grm. of S per litre.

The results of more extended analyses of some samples of urine are given in the following tables, in which the various constituents are expressed in grms. per litre.

TABLE 1.

Animals in the Adelaide District. (Grazing Supplemented by Brewer's Grains.)

Sample. No.	pH.	Density.	Total Nitrogen.	Urea.	Ammonia.	Uric Acid.	Creatinine.	Chloride as NaCl.
1	5·5	1·0386	11·43	7·69	0·317	0·29	1·77	16·85
2	5·6	1·0355	14·08	7·63	0·618	0·36	1·85	11·93
3	5·5	1·0436	12·42	9·45	0·575	0·40	2·41	10·56
4	5·7	1·0151	3·94	2·87	0·492	0·23	0·74	5·64
5	7·4	1·0379	9·09	9·73	0·117	0·31	2·00	10·21
6	6·7	1·0355	10·64	7·77	0·271	0·35	1·71	9·87
7	5·5	1·0350	15·62	9·80	1·126	0·38	1·96	7·30
8	5·6	1·0405	13·07	8·25	0·431	0·37	1·77	8·79
9	5·4	—	8·62	6·22	0·699	0·36	1·41	12·20
Mean	—	1·0352	10·99	7·71	0·516	0·34	1·74	10·37

TABLE 2.

Animals in the Mount Gambier District. (Grazing with Supplement of Meadow Hay.)

Sample. No.	pH.	Density.	Total Nitrogen.	Urea.	Ammonia.	Uric Acid.	Creatinine.	Chloride as NaCl.
1	7·5	1·0333	6·37	5·75	0·046	0·17	0·42	12·24
2	8·0	1·0310	3·86	3·30	0·024	0·13	0·36	10·09
3	7·9	1·0264	2·97	3·26	0·023	0·17	0·27	11·23
4	7·4	1·0142	2·25	2·83	0·038	0·14	0·20	6·65
5	7·9	1·0292	5·56	7·12	0·042	0·32	0·50	13·22
6	7·6	1·0267	2·40	4·84	0·045	0·16	0·27	5·82
7	7·7	1·0373	12·27	8·56	0·084	0·50	0·70	13·83
8	7·8	1·0428	11·63	7·67	0·082	0·55	0·96	19·37
9	7·9	1·0364	10·00	6·54	0·034	0·51	0·68	16·21
10	7·8	1·0192	1·65	2·16	0·015	0·13	0·23	6·32
11	7·7	1·0232	3·00	4·05	0·023	0·10	0·14	7·40
12	7·9	1·0142	1·60	1·63	0·011	0·05	0·11	3·62
Mean	—	1·0278	5·30	4·81	0·039	0·25	0·40	10·50

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